



Using DNA to explore predator diet in temperate marine ecosystems

Kevin Scott Redd

BSc Honours, University of Tasmania

Bachelors in Biology, University of California Santa Cruz

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy.

University of Tasmania

7 April 2017

Declaration of originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of the my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

Kevin Scott Redd

Statement of authority of access

This thesis may be made available for loan and limited copying in accordance with the Copyright Act 1968.

The publishers of the papers comprising Chapters 2, 3, 5 and 7 hold the copyright for that content, and access to the material should be sought from the respective journals. The remaining non published content of the thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968

Kevin Scott Redd

Thesis Abstract

This thesis describes the utility of molecular techniques to detect and identify predator/prey interactions in temperate marine ecosystems with an emphasis on the southern rock lobster, (*Jasus edwardsii*) in Tasmania. A range of DNA-based methods are developed and implemented to better understand the role of this important benthic predator in shaping reef communities. The design and testing of numerous general and species-specific PCR primers is detailed and the utility of these PCR-based assays for monitoring trophic interactions is explored. Captive feeding experiments examine the efficacy of single-species-specific prey detection assays for detection of predation by southern rock lobsters (*Jasus edwardsii*), and also determine the longevity of the DNA signal and the possibility of quantitative PCR to infer the relative amount of prey consumed by these decapod crustaceans. Based upon these results, the molecular methodologies were tested in a large scale manipulative field experiment to investigate the predation capacity of southern rock lobsters on sea urchins (*Centrostephanus rodgersii* and *Heliocidaris erythrogramma*) to understand the shaping of benthic habitats via rock lobster predation in the wild. The results of the dietary component of this experiment are presented and the implications for management are explored briefly. To understand the role of *Jasus edwardsii* in the marine environment, a broad scale molecular prey inventory approach was used to determine the overall diet of southern rock lobsters both spatially at fished and unfished locations and temporally at sites over several years in Tasmania by using molecular cloning and the use of the 454 Next Generation / pyrosequencing platform. The results of these southern rock lobster prey inventories are presented as well as several other examples of situations with other marine animals using related applications of molecular dietary methods where prey information is difficult to obtain (cephalopods) or is problematic due to the challenges of capturing predators (elasmobranchs). Also provided is a critical review of the utility of molecular prey detection in examining the diet of marine crustaceans, cephalopods and chondrychthians and the thesis concludes by summarising the benefits and pitfalls in using DNA-based dietary methods to address both specific predator-prey issues and more general broad scale trophic webs.

Key Words: Molecular prey detection, molecular trophic interactions, prey inventory, rock lobster diet, *Jasus edwardsii*, rock lobsters, sea urchins, *Centrostephanus rodgersii*, *Heliocidaris erythrogramma*, polymerase chain reaction (PCR), quantitative PCR, Real Time PCR, seven gilled shark, *Notorynchus cepedianus*, *Octopus vulgaris*, paralarvae, prey detection

Acknowledgements

These projects came about primarily due to the extensive experience of my supervisors and their ongoing scientific endeavours to further our understanding of the marine environment. The initial work on rock lobsters in particular was the result of discussions between Simon Jarman and Stewart Frusher about the possibility of using the newly emerging molecular techniques to better understand the diet of wild rock lobsters in Tasmania. The sea urchin component began with the fundamental Tasmanian reef ecology work of Craig Johnson and Scott Ling, two of the most enthusiastic and inspiring marine ecologists I have ever met. They both motivated me to think about the complex issues of marine ecosystems in new ways and were the primary drivers for the completion of this thesis. For their support throughout the process I am eternally grateful.

Once the PhD was underway, I was fortunate to meet several other postgraduates who also had interests in these molecular techniques and offered their predators as side projects to tackle. It has been a pleasure to work with Álvaro Roura Labiaga and Adam Barnett on these additional endeavours and to share their enthusiasm when we achieved the results contained in those manuscripts.

All the technical molecular work in this thesis was done at the University of Tasmania's Molecular Genetics Laboratory. This facility was an excellent platform for the development of new techniques as a wide range of researchers from other disciplines also shared the space and their expertise quite freely. In particular, I am indebted to the sage advice of both Dr Bob Elliott and Dr Valérie Hecht whose collective knowledge of molecular biology is incredible. The Laboratory manager, Adam Smolenski also provided ongoing support and assistance as I went along.

The field work has been accomplished in conjunction with the UTAS Marine Ecology (KZA 356) Unit, the *Centrostephanus* project as well as the ongoing sampling which takes place at IMAS/TAFI/MRL. It has been great fun to work alongside the adept and experienced people who also share a passion for the marine environment. In particular David 'Irish' Faloon, Ruari Colquhoun, Kylie Cahill, Sarah Pyke, Dan Haley and the multitude of keen undergraduates who helped us pull lobster pots in the Maria Island Marine Protected Area, as well as at the St Helens and North Bay field sites.

The funding was provided by the Fisheries Research and Development Corporation through two projects; Towards integrated multi-species management of Australia's SE reef fisheries: A Tasmanian example (FRDC 2004/013) and Rebuilding Ecosystem Resilience-Assessment of management options to minimize formation of "barrens" habitat by the long-spined sea urchin (*Centrostephanus rodgersii*) in Tasmania (FRDC 2007/045). Additional funding came from the ANZ Charitable Trust-Holsworth Wildlife Research Endowment.

Of course, none of this would have been possible without the marine organisms which really should be the stars of the show. The southern rock lobster is a very resilient and tenacious creature which I have grown to love over the past few years. I have been accused of keeping these crustaceans as pets, of committing unspeakable acts of torture on them, of cooking them better than anyone else on the planet and of handling them like a commercial fisherman. These highly prized and handsome animals have landed me on the television, in the MoBio 'Where in the World' website and reagent catalogues as well as in the local newspaper on many notable occasions. I honestly hope that the management of these fascinating animals improves in Tasmania and that future generations will know what it is like to hold a fully grown adult *Jasus edwardsii* and to marvel at its beauty.

Table of Contents

Thesis Abstract	3
Acknowledgements	4
Table of Contents	5
Chapter 1: General introduction	10
1.1 Abstract	10
1.2 Introduction.....	10
1.3 Traditional approaches.....	11
1.4 Rise of technology: new approaches.....	12
1.5 Molecular techniques and applications	12
1.6 Overall structure of this thesis	14
Chapter 2 overview	15
Chapter 3 overview	15
Chapter 4 overview	15
Chapter 5 overview	16
Chapter 6 overview	16
Chapter 7 overview	17
Chapter 8 overview	17
Chapter 2: A molecular approach to identify prey of the southern rock lobster.....	19
2.1 Abstract	19
2.2 Introduction.....	19
2.3 Materials and methods	22
Feeding trials	22
Faecal material collection.....	24
DNA extraction	24
PCR amplification.....	25
2.4 Results	27
Feeding trials	27
2.5 Discussion	29
2.6 Acknowledgements	30
Chapter 3: Primer design for molecular prey detection assays	32
3.1 Abstract	32

3.2 Introduction	32
3.3 Materials and methods.....	34
Gene region	34
Genetic databases	35
Sequence selection	36
Primer testing <i>in silico</i>	36
PCR optimization	37
3.4 Results.....	38
3.5 Discussion	39
Chapter 4: Prey quantification and application of real time PCR for molecular prey detection.....	41
4.1 Abstract.....	41
4.2 Introduction	41
4.3 Materials and Methods	46
Feeding experiments	46
Faecal material collection	51
DNA extraction	52
Real time PCR amplification.....	52
4.4 Results:.....	54
4.5 Discussion:	59
Chapter 5: Using molecular prey detection to quantify rock lobster predation on barrens-forming sea urchins	63
5.1 Abstract.....	63
5.2 Introduction	64
5.3 Materials and Methods	66
Experimental sites	66
Sampling rock lobsters.....	67
Faecal material collection	68
DNA extraction	69
PCR amplification.....	69
Filtering PCR amplifications: determining presence of sea urchin DNA.....	70
Analysis of variability in lobster feeding on sea urchins based on assays from field samples	72
Potential passive sources of sea urchin DNA: benthic sediments and excreted sea urchin faeces.....	73
Feeding lobsters benthic sediment/ sea urchin faecal material	73

Lobster predation rates estimated from decline in sea urchin populations.....	75
Estimating change in sea urchin abundance	75
Estimating large lobster abundance.....	77
Estimating predation rates	78
Cross-checking two independent estimates of predation rates	81
5.4 Results	82
Variability in detection rate of sea urchin DNA in lobster faeces	82
‘Extraneous’ sources and passive ingestion of sea urchin DNA	83
DNA based estimates of predation vs. observed sea urchin declines.....	84
5.5 Discussion	84
The potential for molecular detection of predation	84
Interpreting molecular detection of prey.....	86
Limitations of qPCR for determining rates of predation for benthic predators	89
5.6 Acknowledgements	92
5.7 Figures and Tables	93
Chapter 6: Utility of 454 sequencing technology in unravelling predator/prey relationships inside and outside marine protected areas	101
6.1 Abstract	101
6.2 Introduction.....	101
6.3 Materials and Methods	105
Sample collection	105
DNA extraction	107
PCR Amplifications.....	107
Clone library	108
454/Pyrosequencing trials.....	109
6.4 Results	112
6.5 Discussion	121
6.6 Acknowledgements	126
Chapter 7: Applications of molecular prey detection techniques to understand trophic linkages in marine systems.....	129
7.1 Introduction.....	129
7.2 Overall methodology.....	131
7.3 Genetic methodology rationale and overview.....	132
7.4 Molecular methods	134

Sample collection and DNA extraction	134
Specific PCR primer design	137
Genetic databases	138
Polymerase chain reaction (PCR) platform.....	140
Quantification of prey: qPCR	141
Identification of prey from mixed templates: DNA cloning and sequencing	141
Sample bias towards DNA of predators: a pitfall of cloning and sequencing	143
7.5 Pitfalls associated with molecular techniques	144
Primer design <i>in silico</i> based upon sequence availability.....	144
Variability in PCR primer performance.....	145
Secondary predation and ‘background’ material	146
Technical capacity and costs associated with molecular biology.....	147
7.6 Benefits associated with molecular techniques	149
Primers and software are widely available.....	149
High reproducibility and rapid results	149
7.7 Discussion	150
7.8 Conclusion and future directions	155
Chapter 8: The conclusion: resolving marine predator diet on the molecular frontier	159
8.1 Introduction	159
8.2 Conclusion	160
References	162

Chapter 1

General Introduction

“....forever in debt to your priceless advice....”

Heart Shaped Box

Nirvana

In Utero

1993

Chapter 1: General introduction

1.1 Abstract

In this thesis, I describe the use of molecular techniques to detect and identify predator/prey interactions. A range of DNA based methods are explored and compared with more traditional approaches to dietary analysis.

Key Words Molecular prey detection, molecular trophic interactions, prey inventory, rock lobster diet, *Jasus edwardsii*, 454 sequencing, clone library,

1.2 Introduction

The trophic role of predators and their link to food webs is an important question for ecologists (Braccini, 2008, Casper et al., 2007b, Moloney et al., 2011, Rosas-Luis et al., 2014). However, it can be challenging to monitor the behaviour and dietary intake of predators at all times and in all possible locations (Beckerman et al., 2006). Nocturnal predation events can be virtually impossible to observe and animals which reside and forage in complex habitats or remote locations are even more difficult to follow (Deagle et al., 2007, Dalén et al., 2004, Sheppard et al., 2004, McMahan et al., 2013). The marine environment presents additional challenges for research on predator / prey relationships with depth, location, habitat complexity, water temperature and light availability providing just a few of the constraints on understanding and monitoring predation events *in situ* (Passmore et al., 2006, Jarman and Wilson, 2004, Fox et al., 2012, Hudson and Wigham, 2003). Climate related changes to ecosystems, which can lead to shifts in both predator and prey abundance and distribution, are presently of considerable importance (Ling et al., 2009a, Corrigan et al., 2011, Coyle et al., 2011). Monitoring trophic interactions is a valuable method of tracking these changes and in many cases, represents the best possible method of describing ecosystem change and resilience (Jaeger and Cherel, 2011, Pearce-Higgins, 2010). There has been considerable debate over whether 'top down' or 'bottom up' processes are driving ecosystem change (Ling and Johnson, 2009, Christofoletti et al., 2010, Shears and Babcock, 2002, McClanahan and Shafir, 1990). Predation is an extremely important mechanism within the complex functioning ecosystem and therefore prey detection represents a valuable tool in understanding these two theoretical differences which can shed additional light on this long-running debate (Wabnitz et al., 2010).

1.3 Traditional approaches

The study of predator diets has traditionally necessitated a lethal sampling strategy to capture the predator and to remove the gut contents from the animal by dissection (Hyslop, 1980, Pierce and Boyle, 1991). The gut contents must then be examined by visual methods and grouped into the most accurate taxonomic clusters using a microscope or simple sorting protocols (Hyslop, 1980, Hickman, 1945, Light et al., 1983). While this approach can be successfully employed on animals with large fragments of prey in their digestive tracts, or on prey which have hard parts remaining in predator stomachs, there are many groups of animals where visual inspections yield little or no dietary information (Nejstgaard et al., 2003, Williams, 1981). The prey of many invertebrate predators is often visually unrecognizable due to efficient chemical and mechanical digestion as well as the maceration-based feeding style which these animals exhibit (Blankenship and Yayanos, 2005, Symondson, 2002). This is even more challenging when small animals, such as larvae, are the predators in question (Roura et al., 2010, Chow et al., 2006) and the prey are extremely small and often highly degraded by digestion.

In addition, many works describing the diet of predators are based on relatively low sample sizes (Ambrose, 1984, Cortes and Gruber, 1990, Lordan et al., 1998) which may be a result of the collection technique used (Pierce and Boyle, 1991) or the availability of animals to study (Lordan et al., 1998). Although the visual inspection methods presented in these early papers are useful as reference material, there are many species upon which traditional capture and dissection would no longer be feasible (e.g. threatened, endangered and protected species) and a wide range of additional animals from which little dietary information could be obtained using these visual inspection techniques (Mayfield et al., 2000c).

As conservation of species and animal ethics considerations have become increasingly important in designing sampling methods, an entirely new field of prey identification, based upon laboratory techniques, has been developed (Symondson, 2002, Symondson and Liddell, 1995, Kear, 1992, Sint et al., 2011, Waldner and Traugott, 2012). The techniques in this new area employ laboratory-based methods such as protein chemistry, ELISA, stable isotopes, fatty acids and DNA to determine predator diets by methods in addition to or *in lieu* of the traditional dissections and visual inspections (Agusti et al., 2003b, Dalén et al., 2004, Symondson and Liddell, 1995, Mayfield et al., 2000c, Guest et al., 2009, Clare et al., 2009, Mumma et al., 2016, Zarzoso-Lacoste et al.,

2016). Each of these laboratory-based techniques has strengths and weaknesses which are associated closely with the digestive system of the predator, the power and limitations of the laboratory technique and the ecological questions being posed (Mayfield et al., 2000c, Symondson, 2002, King et al., 2008a, Roualdes et al., 2016).

1.4 Rise of technology: new approaches

There are considerable benefits from understanding the role of predators in their environment, in both terrestrial and aquatic systems. The pioneers of modern molecular prey detection were focused on agricultural and fisheries research applications (Zaidi et al., 1999, Chen et al., 2000, Hoyt et al., 2000, Zhu et al., 2000). This is perhaps the reason that these two research areas continue to lead the way in devising new approaches to prey detection in both marine and terrestrial ecosystems (Symondson, 2002, Jarman et al., 2004, Agusti et al., 1999, Lea et al., 2002, Zarzoso-Lacoste et al., 2013, Roslin and Majaneva, 2016, Peters et al., 2015). Of notable importance are the interactions between species in agriculture for the implementation of biological control programs (Agusti et al., 2003b) and in eliminating pest species from native environments (Sheppard et al., 2004, Hoogendoorn and Heimpel, 2001). For marine ecosystems, the primary focus has been on understanding the basic trophic relationships which exist in often remote and poorly studied areas (Passmore et al., 2006, Raclot et al., 1998, Corse et al., 2015). The use of non-invasive sampling techniques, coupled with laboratory analysis has paved the way for an entirely new set of trophic investigations, on animals as small as paralarvae (Roura et al., 2010) and as large as whales (Jarman et al., 2002).

1.5 Molecular techniques and applications

The focus of this thesis is on the development and use of DNA-based assays to better understand the diet of several important marine predators, principally the southern rock lobster (*Jasus edwardsii*) in Tasmania. There are two broad categories into which molecular prey detection assays can be classified; broad/general assays and prey species-specific assays - both of which have been explored in this thesis.

The selection of which assay to use depends largely on the predator being studied, the ecological questions being asked, and any previous knowledge about the potential prey items which may be consumed by the predator (King et al., 2008a, Clare, 2014). For example, a predator may be feeding on just a few species, and therefore a species-specific assay will allow the researcher to determine how important each of these species is in the diet of the predator (Vestheim et al., 2005, Zhu and Greenstone, 1999, Redd et al., 2008, Kartzinel and Pringle, 2015). Directly targeting a relevant ecological question is a valuable way to utilize molecular prey detection techniques (Symondson, 2002, King et al., 2008a). Examples of this include the question of whether larval cod are consumed by other fish species (Rosel and Kocher, 2002), the role of spiders as aphid predators in agricultural areas (Agusti et al., 2003a), the dietary range of bats (Clare et al., 2011), and developing methods to control insects which damage pear crops (Agusti et al., 2003b). In this thesis, the role of the southern rock lobster (*Jasus edwardsii*) in regulating populations of two sea urchin species *Centrostephanus rodgersii* and *Heliocidaris erythrogramma* was explored using species-specific PCR primer sets designed to detect the sea urchin species (Chapter 5). Alternatively, the predator may be foraging on a much wider range of prey which necessitates a broad/generalist approach as shown in salmon preyed upon by seals (Parsons et al., 2005), and arrow squid (Braley et al., 2010). In this thesis, the broad approach was explored by developing group-specific PCR primers (Jarman et al., 2006) for use on any marine predator and by using general/universal PCR primers on unidentified prey of the seven gilled shark (*Notorynchus cepedianus*) (Barnett et al., 2009) and common octopus (*Octopus vulgaris*) paralarvae (Roura et al., 2012) (Chapter 7).

Numerous DNA-based prey detection studies have been developed and performed on such diverse animals as krill, martens, seals, penguins, leopard cats, whales, whale sharks, spiders and a vast array of insects (Read et al., 2006, Jarman and Wilson, 2004, Jarman et al., 2002, Deagle et al., 2009, Passmore et al., 2006, Deagle et al., 2010, O'Meara et al., 2014, Xiong et al., 2016). Much of this work has focused on gaining a general understanding of the role of the predator in the ecosystem and in the ability of the techniques to be applied to the ecological system in question. At present, few studies have undertaken a large-scale temporal and spatial sampling regime to examine specific dietary changes associated with a specific organism, as I have in Chapter 5. In this thesis I also explored the latest developments in 454/pyrosequencing to provide information on spatial and temporal changes in predator diets (Chapter 6). The primary focus of this thesis is the use and evaluation of molecular prey detection techniques to

understand the trophic linkages in marine systems highlighting specific locations to address a key management question of concern.

1.6 Overall structure of this thesis

The molecular techniques in this thesis were devised primarily for the southern rock lobster (*Jasus edwardsii*) to allow for numerous field samples to be collected non-lethally, and to be subsequently processed and efficiently analysed in the laboratory. The development of a non-lethal dietary collection approach enabled large numbers of lobsters to be obtained from Marine Protected Areas (MPAs) and on fisher's vessels as the lobsters could be returned to the reserve or fisher unharmed. In this project large numbers of dietary samples were collected both spatially and temporally and a 96 well format for DNA extraction was determined to be ideal for sample handling and purification as well as for use in a range of downstream applications such as the quantitative Real Time Polymerase Chain Reaction (qPCR) platform. This high throughput molecular system made it possible to analyse a large numbers of samples across broad temporal and spatial scales. This sampling strategy best suited the nature of rock lobster distribution and allowed for material to be collected in a relatively short amount of time by a small research team on board a boat using baited traps, without necessity of hand-capturing each lobster by SCUBA diving.

The specific ecological questions associated with the role of the southern rock lobster on nearshore temperate reefs revolved around several themes: the development of molecular methods to address the diet of the southern rock lobster (Chapters 2, 3 and 4), interaction between rock lobsters and other reef dwelling organisms (Chapter 5), impact of rock lobster removals on the ecosystem (Chapters 5 and 6), differences between fished areas and unfished areas (Chapters 5 and 6), changes in rock lobster diet over time and shifts in diet across size and gender (Chapters 5 and 6). Each of these questions necessitated a different type of molecular assay as a method of exploring the diet and role of the rock lobster and additionally was expanded to other marine organisms to highlight the utility of the techniques (Chapter 7).

Chapter 2 overview

The second chapter of this thesis details the non-lethal method of rock lobster faecal sample collection, and describes the temporal longevity of the molecular signal as a result of feeding experiments developed to assess the utility of species-specific prey detection assays. This chapter has been published as:

Redd, K., Jarman, S., Frusher, S. & Johnson, C. (2008). A molecular approach to identify prey of the southern rock lobster. *Bulletin of Entomological Research- Special Edition Molecular Trophic Interactions* 98, 233-238.

Chapter 3 overview

The third chapter details the method of PCR primer design and the suite of primers which have been designed for use in marine-based prey detection assays. The primer design method outlined in this chapter and the software package of AMPLICON (Jarman, 2004) form the basis of all PCR primers designed and used in this thesis. Portions of this primer design approach have been previously published as follows, but this thesis chapter contains additional species-specific primers designed for use in temperate Australian marine ecosystems:

Jarman, S., Redd, K., & Gales, N. (2006). Group-specific primers for amplifying DNA sequences that identify Amphipoda, Cephalopoda, Echinodermata, Gastropoda, Isopoda, Ostracoda and Thoracica. *Molecular Ecology Notes* 6, 268-271.

Chapter 4 overview

The fourth chapter examines the utility of the sensitive genetic technique, quantitative Real Time Polymerase Chain Reaction (qPCR), to measure the amount of prey DNA in rock lobster faecal samples. Feeding trials in a series of tank-based experiments were undertaken to control the dietary intake of the rock lobsters prior to collection of faecal material for downstream molecular analysis.

Chapter 5 overview

The fifth chapter describes an ecological application of the species-specific prey detection technique to examine the interaction between rock lobsters and potential benthic prey species, *Centrostephanus rodgersii* and *Heliocidaris erythrogramma*, using a large scale controlled experiment in eastern Tasmania. Rock lobsters were introduced to extensive areas of sea urchin 'barrens' and adjacent kelp habitats and these rock lobsters were sampled over time at these locations to determine the extent of rock lobster predation on both sea urchin species using specific sea urchin detecting PCR primers and the quantitative Real Time PCR platform. This chapter has been published in *Molecular Ecology*:

Redd, K., Ling, SD., Frusher, SD., Jarman, SD. and Johnson, CJ. (2014). Using molecular prey detection to quantify rock lobster predation on barrens-forming sea urchins. *Molecular Ecology*, 23(15), 3849-3869.

Chapter 6 overview

The sixth chapter describes a broad molecular approach to look at the entire dietary inventory of rock lobster populations over time with an additional emphasis on spatial variability in diet and the variations in diet between fished and unfished populations of rock lobsters. This chapter covers the lobster dietary samples collected at Maria Island (2005-2010) and at sites in fished locations, the advantages and disadvantages of clone library creation with the use of vectors and plasmids for this purpose, and colony PCR to sequence the results. Additional data were generated by the use of "Next Generation" 454/pyrosequencing and extensive GENBANK searches to match prey species. This chapter is currently being prepared for publication:

Redd, K., Ling, SD., Johnson, CJ., Frusher, SD., Jarman, SD. Utility of 454 sequencing technology in unravelling predator/prey relationships. *Marine Biology*, (In preparation).

Chapter 7 overview

The seventh chapter of this thesis provides an overview of the potential for DNA dietary analysis to address key ecosystem questions from zooplankton to large predators. Specifically, the chapter provides a discussion on the advantages and disadvantages of dietary DNA to improve our knowledge on the trophic relationship of European octopus (*Octopus vulgaris*) paralarvae in the pelagic ecosystem, rock lobsters (*Jasus edwardsii*) in benthic reef ecosystems and the seven gilled shark (*Notorynchus cepedianus*) in coastal bays and estuarine ecosystems. This chapter includes a detailed explanation and critique of the methods used throughout and a guide to using molecular methods for other applications. The additional applications have been published as:

Barnett, A., Redd, K., Frusher, S., Stevens, J. & Semmens, J. (2010). Non-lethal method to obtain stomach samples from a large marine predator and the use of DNA analysis to improve dietary information. *Journal of Experimental Marine Biology and Ecology*, 393, 1-2, 188-192.

Roura, Á., Guerra, Á., Redd, K., González, Á. (2012) Molecular prey identification in wild *Octopus vulgaris* paralarvae. *Marine Biology*, 159, 6, 1335-1345.

Chapter 8 overview

The eighth chapter of this thesis provides a brief conclusion which summarizes key results and highlights the utility of the molecular prey detection techniques which were developed to improve our knowledge on the trophic relationships of temperate marine predators.

Chapter 2:

A molecular approach to identify prey of the southern rock lobster

This chapter has been published as:

Redd, K., Jarman, S., Frusher, S. & Johnson, C. (2008). A molecular approach to identify prey of the southern rock lobster. *Bulletin of Entomological Research- Special Edition Molecular Trophic Interactions* 98, 233-238.

Minor changes to the chapter have been undertaken to ensure consistency in formatting of text, figures and tables.

“...I'm having a daydream, we're getting somewhere...”

Rome wasn't built in a day

Morcheeba

Fragments of Freedom

2000

Chapter 2: A molecular approach to identify prey of the southern rock lobster

2.1 Abstract

I demonstrate the use of molecular techniques to detect specific prey consumed by the southern rock lobster (*Jasus edwardsii*). A quick and non-lethal method was used to collect rock lobster faecal material and a molecular protocol was employed to isolate prey DNA from faecal samples. The isolated DNA was amplified using the polymerase chain reaction (PCR) with PCR primers designed to target specific prey items. Feeding experiments determined that DNA from blacklip abalone (*Haliotis rubra*) and sea urchins (*Centrostephanus rodgersii* and *Heliocidaris erythrogramma*) can be detected in rock lobster faecal samples within seven hours and remains present for up to 60 h after ingestion.

Keywords: *Jasus edwardsii*, *Haliotis rubra*, polymerase chain reaction (PCR), prey detection, rock lobster diet

2.2 Introduction

In many near-shore temperate reef ecosystems, rock lobsters are a dominant predator effecting a top-down control of benthic community structure (Shears and Babcock, 2003, Mayfield and Branch, 2000, Tarr et al., 1996). However, detailed knowledge of rock lobster diets and spatial and temporal variability in diet is poorly understood. This information is crucial to informed management at an ecosystem level of rocky reefs and the important fisheries they support. The establishment of no-take marine protected areas (MPAs), where commercial and recreational fishing is prohibited, has provided a useful resource for monitoring of key temperate reef organisms (Edgar et al., 1997). This has recently led to speculation that southern rock lobster (*Jasus edwardsii*) predation may underpin an apparent decrease in densities of young abalone

within the Tasmanian MPAs (Edgar and Barrett, 1999, Barrett et al., 2003). Currently, there is no method to test this hypothesis or to evaluate the predation patterns of southern rock lobsters in the wild. Furthermore, these are both high value fishery species and, thus, an interesting trophic interaction to monitor.

Previous research on predator-prey relationships of crustaceans has largely involved gut dissection and relied on visual analysis to identify prey remains (Mayfield et al., 2000c, Ennis, 1973, Fielder, 1965, Hickman, 1945, Jernakoff et al., 1993). This approach has shown that the diet of lobsters varies widely with season and that lobsters are selective feeders (Barkai et al., 1996, Joll and Phillips, 1984, Mayfield et al., 2000a, Mayfield et al., 2000b). The physical process of digestion, however, makes visual assessment of gut contents difficult, and gut content analysis has many biases due to varying rates of gut retention, digestion and erosion of prey material. The collection of foregut contents for inspection also necessitates the mortality of the studied lobster (Jernakoff et al., 1993, Joll and Phillips, 1984, Mayfield et al., 2000c, Hickman, 1945, Williams, 1981, Mayfield et al., 2000a) so that large scale surveys are problematic. Killing animals to identify prey is also unlikely to be acceptable if the study population resides in an MPA (Ward et al., 2001). Visual inspection of gut contents is known to miss soft-bodied prey organisms, such as abalone and other gastropod molluscs, and may result in up to 90% of the gut contents being unidentifiable (Mayfield et al., 2000c). Non-lethal techniques to determine dietary intake are essential because of the high value of live rock lobsters in the commercial fishery and to enable ongoing work inside MPAs.

For most marine predators, unbiased identification of prey is problematic, but new protocols are emerging that allow the unambiguous detection of prey species in predator diets. Recent advances in molecular biology have been used to elucidate diets of animals in a wide range of taxonomic groups, such as fish (Rosel and Kocher, 2002), giant squid (Deagle et al., 2005a), wasps (Kasper et al., 2004), spiders (Agusti et al., 2003a) and even introduced insects capable of foraging

in native forests (Sheppard et al., 2004). Molecular techniques take advantage of unique DNA sequences in species or in groups of organisms and can provide detailed and precise information about predator-prey relationships (Juen and Traugott, 2005, Jarman and Wilson, 2004, Deagle et al., 2005a, Nejstgaard et al., 2003, Agusti et al., 2003a).

In the marine environment, protocols have been developed successfully to isolate prey DNA from the faeces of predators (Vestheim et al., 2005, Nejstgaard et al., 2003, Deagle et al., 2005b, Jarman et al., 2004). The DNA is amplified using polymerase chain reaction (PCR) with specific primers that target gene regions of prey species (Sheppard and Harwood, 2005, Jarman et al., 2004). The techniques developed have shown that the identity of prey species can be unambiguously detected in this manner (Symondson, 2002). A major incentive for development of non-invasive techniques as an alternative to direct stomach content analysis has been to allow the non-lethal study of endangered or protected animal species (Jarman et al., 2004, Deagle et al., 2005b, Farrell et al., 2000, Symondson, 2002, Jarman and Wilson, 2004, Jarman et al., 2002).

In Tasmania, lobsters are the basis for a lucrative commercial fishery where fishers land and sell their lobsters live to discerning domestic and international markets. For fishers to support our research, it was necessary to obtain dietary information while still leaving the lobsters in perfect condition for subsequent sale. The capacity to use animals caught in traps was also important to enable researchers to obtain samples aboard commercial rock lobster fishing vessels, to access remote areas of lobster habitat and to obtain animals from deep-water reefs outside standard scuba diving depths.

The aims of this paper were threefold. Firstly, we demonstrated a non-lethal approach to obtaining dietary samples; secondly, we determined if faecal material could be used to identify prey using molecular methods; and, thirdly, we determined if the longevity of the DNA signal was sufficient to include prey items from lobsters that would be retained in traps for up to 24 h.

2.3 Materials and methods

Feeding trials

Rock lobsters for feeding trials were captured by scuba diving in the Crayfish Point Marine Reserve at Taroona, Tasmania (42.95°S, 147.34°E) in April 2004 and May 2005. Lobsters were collected opportunistically, ensuring an even distribution of sexes and a wide range of sizes. All captured lobsters were measured and carapace length (CL) was recorded to the nearest millimeter.

Captured lobsters were immediately taken to the laboratory and kept in aerated, flow-through seawater tanks. For the duration of the feeding trials, lobsters were maintained under ambient light conditions and water temperatures in outdoor aquaria at the Marine Research Laboratories, Taroona, Tasmania.

For each trial, the lobsters were placed in one section of a 450 l tank separated into three sections with plastic mesh and dividers. Each lobster was provided with a 400 mm×200 mm concrete block as a shelter. All lobsters were starved for 72 h prior to each feeding trial to facilitate gut evacuation and to remove any remaining prey DNA from the digestive tract. For each trial, approximately 15 g of fresh food material was given to each lobster. This material was prepared by shucking live abalone (*Haliotis rubra*) and cutting the foot tissue into approximately 15 g portions. Common sea urchins (*Heliocidaris erythrogramma*) and long-spined sea urchins (*Centrostephanus rodgersii*) were shucked alive and approximately 15 g portions of roe and viscera were used as food. Food was introduced to each lobster at 1700 h and individual lobsters were monitored for feeding activity. Only lobsters that actively fed and consumed the entire food sample within the first hour were used in the feeding trials. No additional food was provided to lobsters for the duration of the trial and each lobster was sampled once per feeding trial. Lobsters

were selected for faecal collection over the next five days at the times (hours after commencement of feeding) given in table 1. Lobsters were selected randomly to eliminate any tank effect. For each sampling time, faecal material was collected from a minimum of three separate lobsters. For each of the individual faecal samples, PCR assays were performed twice to guarantee the consistency of the result. These feeding experiments were repeated over a period of 65 days with 116 faecal samples collected in total. A total of 61 lobsters were used in these trials, comprising 24 females and 37 males. Lobsters ranged in size from 53 to 161 mm carapace length.

Table 1. Feeding trial schedule over five days, covering 96 h after initiation of feeding episodes. Each time indicates when samples were collected and the hours after the commencement of feeding.

Day	Sampling time (hours after commencement of feeding)
Day 1	2000 (3 h), 2200 (5 h)
Day 2	0000 (7 h), 0200 (9 h), 0400 (11 h), 0600 (13 h), 0800 (15 h), 1000 (17 h), 1200 (19 h), 1400 (21 h), 1700 (24 h), 2300 (30 h)
Day 3	0500 (36 h), 1100 (42 h), 1700 (48 h), 2300 (54 h)
Day 4	0500 (60 h), 1100 (66 h), 1700 (72 h), 2300 (78 h)
Day 5	0500 (84 h), 1100 (90 h), 1700 (96 h)

Faecal material collection

A 'cradle' was designed and built for the purpose of restraining lobsters while samples could be taken from the hindgut region. Lobsters were placed in the cradle, which holds the animal upside-down and immobilizes the posterior four pairs of walking legs. The anterior telson was held firmly by the researcher to stabilize the abdominal region and tail while collecting faeces.

Lobster faeces were collected using a 100–1000 µl pipette with disposable tips. For each faecal sample, a new sterile tip was used to prevent contamination between samples. The tip was inserted directly into the anal pore of the lobster to remove faeces from the hindgut region. The collected material was immediately pipetted into a 1.5 ml micro centrifuge tube containing 500 µl of 70% ethanol. Ethanol has been shown to be an effective preservative for field samples and does not require freezing or any special handling (Jarman et al., 2004). The volume collected varied from approximately 10 µl to 1 ml depending on the size of lobster and fullness of hindgut at the time of sampling. Ethanol was removed from samples before DNA extraction by centrifuging at 10,000 g for 30 s. Excess ethanol was poured off and the sample tubes centrifuged again. Any remaining ethanol was then removed by pipette.

DNA extraction

There were no previously published studies using rock lobster faecal material as a source of prey DNA. Tissue samples from predator and prey species were collected to test PCR primer specificity and to use as both positive and negative DNA controls in later experiments. Tissue samples were taken from fresh animals and a small (~0.25 ml) portion was used for total cellular DNA extraction. Another portion (~2 ml) of the tissue was stored in 70% ethanol as a voucher specimen. The DNeasy® Tissue Kit (QIAGEN) was used to extract genomic DNA from predator and prey tissues, and the manufacturer's animal tissue protocol was followed.

The Ultra Clean™ Fecal DNA Kit (Mo Bio Laboratories, Inc.) was used for DNA extractions on rock lobster faecal samples following the manufacturer's protocols with the supplied proprietary buffers and reagents. All DNA extracted from faecal samples using this kit was ready for PCR, and the manufacturer's protocol appeared to remove any potential PCR inhibitors.

PCR amplification

Precautions were taken during preparation of PCR reactions to minimize the possibility of contamination by extraneous DNA. Aerosol-resistant barrier pipette tips were used for preparing all PCR reactions, and pipette tips were either sterile and pre-packaged or autoclaved prior to use. All PCR reactions were prepared in a dedicated hood where PCR tubes, pipettes and pipette tips were subjected to UV light for a minimum of 10 min prior to setting up each PCR reaction.

The components of the 20 µl PCRs were 50 mM KCL, 15 mM Tris.HCl pH 3.0, 5.0 mM MgCl₂, 100 µg ml⁻¹ bovine serum albumin, 0.5 mM of each dNTP, 2 mM each primer (Geneworks), 1 unit AmpliTaq Gold® DNA polymerase (Sigma), and ~20 ng template DNA. Both positive and negative controls were run with each batch of PCRs. For negative controls, 2 µl MilliQ H₂O was used as template; and, for positive controls, ~20 ng template DNA from *Haliotis rubra*, *Centrostephanus rodgersii* and *Heliocidaris erythrogramma* was used to confirm reaction success. PCR replication was performed by running reactions a minimum of two times to ensure a consistent result.

The PCR primer sets used in this experiment (Table 2) were developed previously to distinguish *Haliotis rubra* tissue from that of other *Haliotis* species for forensic purposes (Elliott et al., 2002) and for identification of echinoderms as prey items in marine ecosystems (Jarman et al., 2006). Primers were obtained from GeneWorks Pty Ltd Custom Oligonucleotide service and diluted to 10 µM for use in setting up PCR reactions.

PCR thermal cycling conditions for HalCO2GENA and HalCO2GENB primers were: denaturation and DNA polymerase activation at 95°C for 10 min followed by 10 initial amplification cycles: 94°C for 30 s, annealing at 60–55°C for 30 s, 72°C for 1 min with a decrease in the annealing temperature by 0.5°C for each of the 10 cycles. Twenty-five further amplification cycles were carried out: 94°C for 30 s, 55°C for 30 s and 72°C for 1 min. The final extension step was 72°C for 5 min and the reaction was held at 10°C until removed from the MJ Research PTC-2001 Thermal Cycler.

PCR thermal cycling conditions for EchinNSS18sf/EchinNSS18sr primers were: denaturation and DNA polymerase activation at 95°C for 10 min followed by 35 amplification cycles: 95°C for 5 s, annealing temperature at 51°C for 30 s, 72°C for 30 s. The final extension step was 72°C for 5 min and the reaction was held at 10°C until removed from the MJ Research PTC-2001 Thermal Cycler.

Table 2. PCR primers used, including sequence of each primer, target organism or group, and DNA region amplified. DNA regions are mitochondrial cytochrome oxidase subunit I (mt COI) and the nuclear large subunit ribosomal RNA gene (18s rDNA).

Primer	Sequence (5'–3')	Target species/group	DNA region
HalCO2GENA	CAA TYT GAA CYA TTC TMC CAG C	<i>Haliotis rubra</i>	mt COI
HalCO2GENB	CCT TAA ART CTG AGT ATT CGT AGC C	<i>Haliotis rubra</i>	mt COI
EchinNSSf1	GCG TGC TTT TAT TAG GA	Echinodermata	18s rDNA
EchinNSSr1	CGA CCA TGR TAR GCG CAT AAC G	Echinodermata	18s rDNA

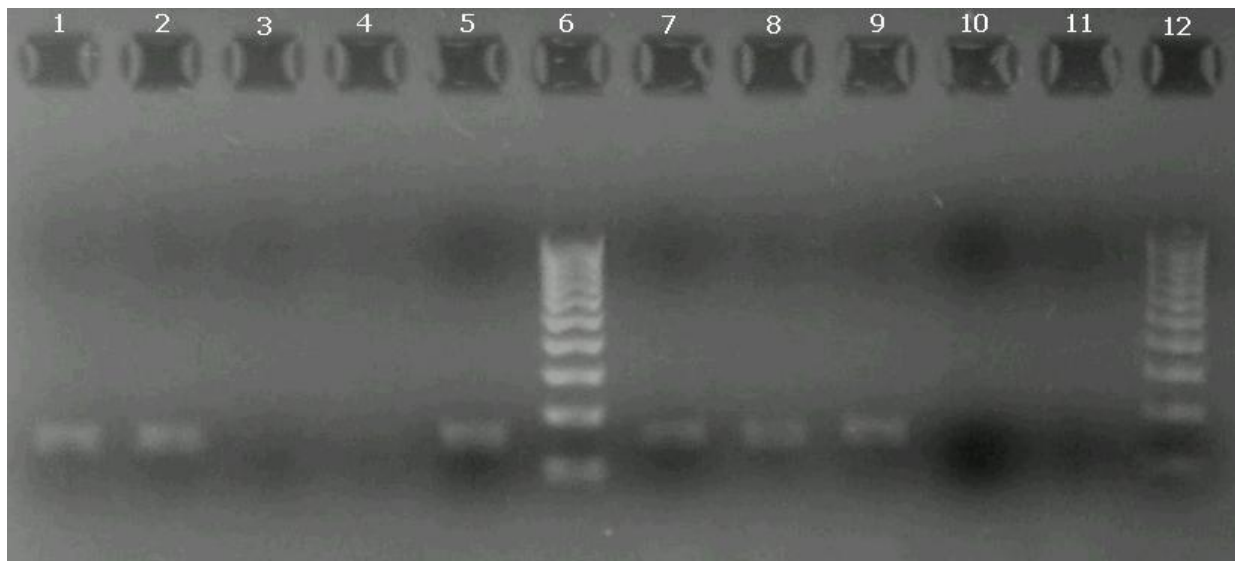
PCR products were visualized by electrophoresis on 1.5% agarose gels stained with ethidium bromide (5 µl ethidium bromide per 100 ml agarose). Each gel was loaded with 7 µl of PCR product, and a 100 bp ladder was used on every gel to determine fragment size and to confirm the results of each PCR. All agarose gels ran for 20 min at 80 V.

2.4 Results

Feeding trials

Using HalCO2GENA and HalCO2GENB primers, fragments of 193 bp were successfully amplified from both black-lipped abalone tissue DNA and faecal samples from lobsters fed a diet of black-lipped abalone (fig. 1). Using EchinNSSf1 and EchinNSSr1 primers, fragments of 160 bp were successfully amplified from *Centrostephanus rodgersii* and *Heliocidaris erythrogramma* tissue DNA, and from faecal samples from lobsters fed a diet of both urchin species (fig. 1). Prey was initially detected in all samples at 7 h after feeding and was consistently detected in all samples until 60 h after the feeding episode. There were no detections in any of the samples taken at 3 and 5 h or for the six sampling times from 66 h to 96 h after commencement of feeding.

Fig. 1. Specificity of HalCO2GENA/HalCO2GENB and EchinNSSf1/EchinNSSr1 PCR primers. Agarose gel showing PCR products amplified from rock lobster feeding experiments using group and species-specific PCR primers. Lanes 1–5 show DNA amplification using PCR primers specific to *Haliotis rubra* and lanes 7–11 show DNA amplification using PCR primers specific to Echinodermata. Lanes 6 and 12 are 100 bp DNA reference ladders indicating relative size of DNA fragments in sample lanes. Lanes 1, 2 and 3 indicate PCR amplified rock lobster faecal samples from 8, 24 and 96 h after feeding on black-lipped abalone. Lane 5 shows DNA amplification using *Haliotis rubra* tissue. Lanes 7, 8, 9 and 10 show PCR amplified rock lobster faecal samples from 12, 36, 48 and 72 h after feeding on *Centrostephanus rodgersii*. Lanes 4 and 11 are negative controls with water instead of DNA sample template.



2.5 Discussion

The faecal collection technique developed met the important requirement of being non-lethal. During the feeding trials, several individual lobsters were sampled ten times over four months using the non-destructive faecal collection technique with no obvious deleterious impacts. The ability to repeatedly sample the same individual lobster confirms the low impact nature of this method and allows for a greater range of experimentation on lobster diet and feeding behaviour. Faecal material was easily and successfully extracted from both sexes of lobsters and a wide range of size classes (53 mm CL-161 mm CL). This indicates that faecal material is useful to study ontogenetic dietary shifts and dietary changes associated with maturity and reproduction.

Molecular prey detection depends upon the ability of DNA to resist digestion in the predator gut and on the power of PCR to amplify a prey specific region of DNA from semi-digested material (Deagle et al., 2005b, Nejstgaard et al., 2003, Parsons et al., 2005, Jarman et al., 2002). The extent of DNA breakdown after digestion by rock lobsters was previously unknown, although Mayfield et al. (2000c) found that digestion rendered the use of serological methods unsuitable. This study shows, for the first time, that prey DNA survives digestion, can be isolated from lobster faecal material and can be successfully amplified using PCR.

The longevity of the molecular signal indicates that samples obtained from traps during routine fishing operations would retain prey DNA consumed prior to entering the trap. In *Jasus edwardsii* fisheries, traps are set for a maximum of 24 h. These results suggest that dietary information should still be available from faecal material for at least the 24 h prior to entering the trap as the dietary signal was recorded in all samples up to 60 h after the commencement of feeding.

For animals such as rock lobsters where previous dietary studies have been inconclusive, DNA holds considerable promise for assessing specific prey variability across spatial and temporal scales. Although rock lobsters and abalone are common on temperate reefs around the world

(e.g. South Africa, New Zealand, Australia), there are no records of the presence of abalone in rock lobster dietary studies (Mayfield and Branch, 2000, Tarr et al., 1996, Hickman, 1945, Mayfield et al., 2000c). Commercial abalone divers often report empty abalone shells in front of lobster dens. The failure of previous studies to identify abalone in lobster guts is possibly due to the 'foot' tissue being the only part consumed by rock lobsters and without any shell fragments, this tissue would be unidentifiable. Understanding the interaction between these two valuable recreational and commercial species is one example of the potential of DNA dietary studies for improving our understanding of marine ecosystems. Importantly, the ability to obtain dietary information non-destructively enables comparisons between fished and non-fished (e.g. MPAs) regions, to better understand the trophic impacts of harvesting marine resources.

2.6 Acknowledgements

This research was a component of grant 2004/013 'Towards integrated multi-species management of Australia's SE reef fisheries: A Tasmanian example', which is supported by funding from the Fisheries Research and Development Corporation on behalf of the Australian Government. Funding was also provided by the ANZ Charitable Trust Australia's Holsworth Wildlife Research Endowment. We would additionally like to thank all the individuals at the Marine Research Laboratories who assisted with rock lobster husbandry and the collection of lobsters and prey species. All experiments were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 7th Edition 2004.

Chapter 3

Primer design for prey detection assays

Portions of this primer design approach have been published as:

Jarman, S., Redd, K., & Gales, N. (2006). Group-specific primers for amplifying DNA sequences that identify Amphipoda, Cephalopoda, Echinodermata, Gastropoda, Isopoda, Ostracoda and Thoracica. *Molecular Ecology Notes* 6, 268-271.

“...I think we've half a chance,
judging by all of the people that I've met...”

My Best Mistake

The Panics

Sleeps Like a Curse

2005

Chapter 3: Primer design for molecular prey detection assays

3.1 Abstract

All PCR-based molecular assays require primers to facilitate the amplification of target DNA sequences *in vitro*. The design, use and implementation of these primers are therefore critical to the success of any assay and the primer design process is described in detail in this chapter. Here we explore the methods of choosing a gene region, using genetic databases, selecting appropriate conserved and unique sequences upon which to base primers, testing of primers and ultimately utilizing the primers in molecular prey detection assays. Portions of this primer design approach have been previously published, but this chapter contains additional species-specific primers designed for use in temperate Australian marine ecosystems.

Key Words Prey detection, PCR primer design, species-specific PCR primers, group-specific PCR primers

3.2 Introduction

The laboratory protocols for isolating, amplifying and sequencing DNA from animal tissues and prey items are well documented and can be easily followed in any facility with basic molecular capabilities (Hebert et al., 2003a, King et al., 2008b). The process of species identification by PCR and DNA sequencing is a rapidly expanding field of research (Ward et al., 2005, Symondson, 2002, Muraji et al., 2004, Pineau et al., 2005, Ratnasingham and Hebert, 2007, Zarzoso-Lacoste et

al., 2013) and a wealth of genetic sequences are now available for DNA identifications from a wide range of species. There are stringent standards for sequence submission to the international genetic databases with procedures to facilitate accurate identifications to the highest taxonomic resolution (Hebert et al., 2003b).

Because PCR primer design is critical to every amplification-based assay, there are numerous software packages available for designing primers such as QuantPrime, PRISE, and Oligo7 (Chen et al., 2002, Arvidsson et al., 2008, Fu et al., 2008, Rychlik, 2007). Most of these programs focus on a single DNA sequence and base the primer design only on that sequence. This sort of primer design is useful in situations where only a single specific DNA template is to be targeted and there is unlikely to be any other DNA available to be amplified by the same primer set. The most obvious example of this would be the design of PCR primers for use in human or mouse genetics, as there is little chance of any other mammalian DNA in a mouse or human blood specimen collected under laboratory conditions.

In the case of predator/prey experiments, there are at least two different sets of DNA templates present in each sample- the prey species as well as the predator itself. There is also the possibility of multiple prey species being present in any given stomach or faecal sample, as well as a potential range of endosymbiotic microbes and this mixed template situation necessitates an approach where only specific prey sequences are amplified, and predator sequences are not (King et al., 2008a). For these situations, it is imperative to have *a priori* knowledge of the predator and prey species in the system and to use a program such as AMPLICON (Jarman, 2004). AMPLICON is a free program for designing PCR primers which will amplify a given set of sequences but not another set of sequences.

In most molecular predator/prey experiments there are two main types of PCR primers which can be used; 'universal primers' and 'group or species-specific primers'. Universal primers facilitate the amplification of a wide range of DNA templates by binding to conserved regions of target species during the PCR. Universal primers are most commonly developed for the amplification of a very broad range of organisms, such as all eukaryotes (Blankenship and Yayanos, 2005, Folmer et al., 1994, Pineau et al., 2005), or all prokaryotes (Bowman and McCuaig, 2003, Dorigo et al., 2002). The nature of these primers lend themselves to prey detection assays where it is necessary to identify unknown material from discrete samples such as chunks of prey material or to resolve a complex mixed sample such as faeces or vomit (Kasper et al., 2004). Group or species-specific primers are designed to amplify only DNA that is specific to selected groups of species or individual species (Zeale et al., 2011, Jarman et al., 2006, Jarman et al., 2004, King et al., 2010). These primers facilitate testing of ecological hypotheses and are particularly useful when screening larger numbers of dietary samples (Admassu et al., 2006, King et al., 2010, Rougemont et al., 2004, Vestheim et al., 2005).

3.3 Materials and methods

Gene region

The mitochondrial 16S ribosomal RNA gene (16S rRNA) has been used successfully for designing group specific primers to amplify small regions of DNA which are highly informative and well suited to molecular dietary analysis (Deagle et al., 2005a, Deagle et al., 2007, Jarman et al., 2004, Braley et al., 2010, Kasper et al., 2004). For these reasons, it is recommended that primers target small, multi-copy DNA fragments from prey material when designing universal primers for the 16S rRNA gene (Simon et al., 1994) or group-specific primers which will anneal to short target

DNA templates of potential prey items. The cytochrome C oxidase subunit I gene (CO1, COa, COb) also meets these criteria (multi-copy, small fragments, well sequenced) which makes it appear potentially useful for the design PCR primers, but there appear to be a greater proportion of highly conserved sequences within this region and only minor variations of single nucleotides between species and divergent groups of organisms. For these reasons, it is not recommended that the cytochrome oxidase region be chosen as a basis for designing species-specific PCR primers as the opportunities for incorrect amplifications and non-specific binding exist, and it can be challenging to find a primer binding site in this region which is truly species-specific. Cytochrome oxidase is much more conducive to barcoding applications and as the basis for universal primer design. For prey detection applications where large pieces of unidentified prey items are available for DNA isolation, amplification and sequencing, the Cytochrome oxidase region is well suited (Barnett et al., 2009).

Genetic databases

Both the National Center for Biotechnology Information (NCBI) GenBank and the Barcode of Life (BOL) project have emerged as the most important global repositories for species-specific DNA sequences and these databases provide a wealth of information for all fields of science (Hebert et al., 2003b, NCBI, 2006, Benson et al., 2008). There are many other smaller databases which cater to more specific research communities, but these more specialist databases such as FlyBase for *Drosophila* genes and genomes (Tweedie et al., 2009) or Mammalian Gene Collection for human, rat, mouse and cow genes (Strausberg et al., 1999) are not necessarily appropriate tools to design PCR primers for prey detection assays. The NCBI GenBank presently has a greater range of sequences housed, but the Barcode of Life has gained considerable momentum in recent years and BOL has much more stringent procedures for the taxonomic identification of submitted samples. For any molecular prey detection assay or primer design application it is highly

advisable to check both of these databases before proceeding with primer design or identification of unknown prey sequences.

Sequence selection

The program AMPLICON was used exclusively for the purpose of designing PCR primers which would amplify a specific species (or group of species) but not another group of species. The design of single species prey detection primers for southern temperate reefs in Tasmania was based upon the initial *a priori* knowledge of the rock lobster diet and a few key organisms were chosen (Table 1 and Table 2). As many other potential prey species were also found on the (NCBI) GenBank and the Barcode of Life databases, 16s sequences from these species were aligned using AMPLICON. All potential prey species were sorted into the Excluded Group in AMPLICON and the prey species of interest was relegated to the Target Group. Gene regions from each potential Target Group (prey species for which the primers were being designed) were then selected using the Primer for Forward Site and Primer for Reverse Site utilities.

Primer testing *in silico*

Although numerous primers can be created *in silico*, the primer sequence is only one component of the molecular prey detection assay development process. There are many additional considerations about the reaction components and the compatibility of primer sequences to actually facilitate the PCR reaction and these must be tested empirically. Primers should ideally have a melting temperature (T_m) between 65°C and 70°C with the actual annealing temperature used during PCR approximately 10°C to 15°C less than the predicted T_m . Primers should be between 12 and 18 bases long and ideally contain approximately equal numbers of each

nucleotide type, without long runs of the same nucleotide. To increase the specificity of the primer, the 3' end should terminate in a C or an A nucleotide. It is better if the 3' end has more A and T, as it will be less prone to mispriming during the PCR amplification. It is critical to avoid internal hairpin structures and self-complementarity which can result in primers which will anneal to each other, forming primer-dimers instead of binding to target template DNA. Most primer design software including AMPLICON (Jarman, 2004) are equipped with desktop utilities to test for self-complementarity, hairpins and T_m before the primers are actually synthesized.

PCR optimization

When optimizing a PCR reaction, there are numerous reagents required and altering the concentrations of these reagents in the reaction mixture will greatly impact on the efficiency of the PCR. Most PCR reactions occur in a buffer which is often a proprietary formulation recommended (and provided) by the supplier of the DNA polymerase and associated reagents. Subtle differences in concentration of buffer components, especially magnesium chloride can greatly impact on the success of the PCR.

Although most PCR primer design software contains a utility for predicting the melting temperature and therefore the annealing temperature at which the primers will perform ideally, it is still necessary to test a relatively broad range of these temperatures for each newly designed primer set. This is commonly carried out by using a PCR thermocycler equipped with a gradient feature. PCR reactions are set up and tested concurrently using a range of different annealing temperatures, and the resulting amplicons are subsequently assessed for presence, quality and quantity by agarose gel electrophoresis or similar methods.

3.4 Results

There are many universal, group-specific and species-specific PCR primers which are appropriate for use in predator/prey assays, some of these primers are listed in Tables 1-2.

Table 1. PCR primers used and developed in this thesis including; primer name and sequence, DNA region targeted by primer, level of taxonomic specificity, target organism or group of organisms, approximate size of amplified DNA fragment (amplicon) and original publication where primers are described.

Primer Name	Primer Sequence 5'–3'	Target DNA Region	Specificity	Target species/group	Amplicon Size	Publication
LR-N-13398 (16s ar)	CGC CTG TTT ATC AAA AAC AT	16s	Universal	Invertebrates	500-650	Folmer, et al 1994
LR-J-12887 (16s br)	CCG GTC TGA ACT CAG ATC ACG T	16s	Universal	Invertebrates	500-650	Folmer, et al 1994
FishF1	TCA ACC AAC CAC AAA GAC ATT GGC AC	COI	Class	Teleost and Chondrichthyes	655	Ward, et al 2005
FishR1	TAG ACT TCT GGG TGG CCA AAG AAT CA	COI	Class	Teleost and Chondrichthyes	655	Ward, et al 2005
FishF2	TCG ACT AAT CAT AAA GAT ATC GGC AC	COI	Class	Teleost and Chondrichthyes	655	Ward, et al 2005
FishR2	ACT TCA GGG TGA CCG AAG AAT CAG AA	COI	Class	Teleost and Chondrichthyes	655	Ward, et al 2005
HalCO2GENA	CAA TYT GAA CYA TTC TMC CAG C	COII	Species	<i>Haliotis rubra</i>	193	Conod, et al 2002
HalCO2GENB	CCT TAA ART CTG AGT ATT CGT AGC C	COII	Species	<i>Haliotis rubra</i>	193	Conod, et al 2002
EchinNSSfl	GCG TGC TTT TAT TAG GA	18s	Phylum	Echinodermata	160	Jarman, et al 2006
EchinNSSrl	CGA CCA TGR TAR GCG CAT AAC G	18s	Phylum	Echinodermata	160	Jarman, et al 2006
GastNLSfl	GCG GYA ACG CAA ACG AAG T	18s	Phylum	Cephalopoda	188-198	Jarman, et al 2006
GastNLSrl	CGA AAW TMA CAC CGT CTC TCC G	18s	Phylum	Cephalopoda	188-198	Jarman, et al 2006
16Sferu	GAC GAT AAG ACC CTA TAA	16s	Superorder	Eucarida	194-204	Roura, et al 2012
16Sferu	CGC TGT TAT CCC TAA AGT AA	16s	Superorder	Eucarida	194-204	Roura, et al 2012
Helio 16sF	TCA AAG GAA GTT ACC G	16s	Species	<i>Haliotidaris erythrogramma</i>	202	Redd, 2004
Helio 16sR	CCC TTA AAA GCT TCT GCA CCC T	16s	Species	<i>Haliotidaris erythrogramma</i>	202	Redd, 2004
Aba2F	GCT TTT GCT GGA AAA ACT CG	16s	Species	<i>Haliotis rubra</i>	235	Redd, 2004
Aba2R	CGT TCC CCA GTT TTA GGT GA	16s	Species	<i>Haliotis rubra</i>	235	Redd, 2004
Centro 16sF	GGA ACA GCA AAC ATG GAG AGT CCT GC	16s	Species	<i>Centrostephanus rogersii</i>	161	Redd, 2004
Centro 16sR	CCG TCT TGC CAT TCA TGC CAG TCT CTA	16s	Species	<i>Centrostephanus rogersii</i>	161	Redd, 2004
CENT 2 F	GGG CAA CCG CGG AGA AGG AAT ATC C	16s	Species	<i>Centrostephanus rogersii</i>	268	Redd, 2004
CENT 2 R	CGC AAA CCT TTT TGT CAA	16s	Species	<i>Centrostephanus rogersii</i>	268	Redd, 2004
Hali16sF	GGG GTG ACT GGG GAA CAA T*AGT	16s	Genus	<i>Haliotis</i> sp.	149	Redd, 2004
Hali16sR	CTA CAC CCT CAG GAC ACC TTA ATCC	16s	Genus	<i>Haliotis</i> sp.	149	Redd, 2004
Plagl16sF	GAC *GT GCA AAG GTA GCA TA	16s	Genus	<i>Plagusia</i> sp.	400	Redd, 2004
Plagl16sR	TAA TTC AAC ATC GAG GTC GCA	16s	Genus	<i>Plagusia</i> sp.	400	Redd, 2004
Pallidus16sF	CTC GGT GAG ATA TAA	16s	Species	<i>Octopus pallidus</i>	147	Redd, 2004
Pallidus16sR	GTC CCT TTA A	16s	Species	<i>Octopus pallidus</i>	147	Redd, 2004
Maorum16sF	GCT CGG TGA GAA TAA AAT	16s	Species	<i>Octopus maorum</i>	173	Redd, 2004
Maorum16sR	GTC CCT TTA AA	16s	Species	<i>Octopus maorum</i>	173	Redd, 2004
Jas16sF	TCA AAT ATC CT* GGG GGA CGA TAA GAC CCT ATA	16s	Species	<i>Jasus edwardsii</i>	259	Redd, 2004
Jas16sR	CCT TGC CTT CGA TAA GGA CTC TC	16s	Species	<i>Jasus edwardsii</i>	259	Redd, 2004

=

Table 2. PCR primer testing on nine pairs of PCR primers developed for this thesis, including a detailed taxonomy of the species on which primers were tested. Results of primer tests are (+) for positive amplification of this species and (-) for negative amplification of the species.

Phylum	Class	Order	Family	Species	Primers LS-1/2SR/1 LS-46/2SR	Primers Helo 36R/ Helo 36R	Primers Rho2T/ Rho2R	Primers Centro 36R/ Centro 36R	Primers CENT 2 S/ CENT 2 R	Primers Holo36R/ Holo36R	Primers Pug2T/ Pug2R	Primers Pall36R/ Pall36R	Primers Muss36R/ Muss36R	Primers Jus36R/ Jus36R
Crustacea	Malacostraca	Decapoda	Palinuridae	<i>Jaesius edwardsii</i>	+	-	-	-	-	-	-	-	-	+
Crustacea	Malacostraca	Decapoda	Plagusidae	<i>Plagusia chabrus</i>	+	-	-	-	-	-	+	-	-	-
Crustacea	Malacostraca	Decapoda	Portunidae	<i>Nectocarcinus integrifrons</i>	+	-	-	-	-	-	-	-	-	-
Mollusca	Cephalopoda	Octopoda	Octopodidae	<i>Octopus maorum</i>	+	-	-	-	-	-	-	-	+	-
Mollusca	Cephalopoda	Octopoda	Octopodidae	<i>Octopus pallidus</i>	+	-	-	-	-	-	-	+	-	-
Mollusca	Gastropoda	Orthogastropoda	Haliotidae	<i>Haliotis rubra</i>	+	-	+	-	-	+	-	-	-	-
Mollusca	Gastropoda	Patellogastropoda	Acanthidae	<i>Notoacmaea petterdi</i>	+	-	-	-	-	-	-	-	-	-
Mollusca	Gastropoda	Caenogastropoda	Muriceidae	<i>Thais orbita</i>	+	-	-	-	-	-	-	-	-	-
Mollusca	Gastropoda	Archaeogastropoda	Trochidae	<i>Austrocochlea constricta</i>	+	-	-	-	-	-	-	-	-	-
Mollusca	Gastropoda	Archaeogastropoda	Turbinidae	<i>Turbo undulatus</i>	+	-	-	-	-	-	-	-	-	-
Mollusca	Gastropoda	Littorinomorpha	Littorinidae	<i>Africollitona praeternissae</i>	+	-	-	-	-	-	-	-	-	-
Echinodermata	Echinoidea	Echinoidea	Echinometridae	<i>Helicodonta erythrogramma</i>	+	+	-	-	-	-	-	-	-	-
Echinodermata	Echinoidea	Diadematoida	Echinometridae	<i>Centrostephanus rogersii</i>	+	-	-	+	+	-	-	-	-	-

3.5 Discussion

Although developing both species-specific and group-specific PCR primers can be slightly challenging where complete sequence coverage is not available, we provide solutions to prey detection issues in this chapter. Group-specific PCR primers were designed to amplify the most common potential prey species from a wide range of marine taxa based upon available sequence data from the variable 3' end of the mitochondrial ribosomal 16s gene region. This region is widely used for both molecular identification and dietary analysis.

Any new molecular prey detection assay may necessitate the design of specific PCR primers for the monitoring of trophic interactions. The PCR primers presented in this chapter will detect a broad range of temperate marine invertebrates and may additionally be used to identify or to quantify larvae from water samples or other unknown environmental material.

Chapter 4

Prey quantification and application of real time PCR for molecular prey detection

“Do you believe that there are treasures in the ocean?

...No lonely hands grab my suitcase full of nothing...

I don't know why...”

Just a Boy

Angus and Julia Stone

A Book Like This

2007

Chapter 4: Prey quantification and application of real time PCR for molecular prey detection

4.1 Abstract

We apply molecular techniques to detect and quantify specific prey consumed by the southern rock lobster (*Jasus edwardsii*) under experimental conditions. A quick and non-lethal method was employed to collect rock lobster dietary samples which were screened for the presence of sea urchins (*Centrostephanus rodgersii* and *Heliocidaris erythrogramma*) and abalone (*Haliotis rubra*) using the quantitative real time PCR (qPCR) platform. Additional environmental samples from benthic sediments collected at marine research sites where urchin barrens were documented in both full and incipient conditions were examined to determine the amount of prey DNA present in the marine environment.

Key Words *Jasus edwardsii*, rock lobsters, sea urchins, *Centrostephanus rodgersii*, *Heliocidaris erythrogramma*, abalone, *Haliotis rubra*, polymerase chain reaction (PCR), Real Time PCR, prey detection, rock lobster diet

4.2 Introduction

In many near-shore temperate reef ecosystems, rock lobsters are a dominant invertebrate predator but their role in shaping benthic community structure has been widely debated (Andrew and MacDiarmid, 1991, Tegner and Levin, 1983a, Waddington et al., 2008). There are presently few methods of unravelling the role of rock lobsters *in situ*, but an abundance of unsubstantiated anecdotal evidence has been reported about what these important predators do on the benthos (Barkai et al., 1996, Denny, 2010). Fishers have a commonly held belief that rock lobsters are

strictly scavengers, taking primarily detritus and dead material from rocky reef environments (Treloggen, 2009, Hickman, 1945). This belief was most likely developed by fisherman who commonly use dead fish as the main type of bait for attracting rock lobsters into their pots (Treloggen, 2009). The more likely scenario is that rock lobsters are, in fact, opportunistic predators, encountering a wide range of prey species and consuming items where ontogenetically appropriate (Mayfield et al., 2000a, Mayfield et al., 2000b, Guest et al., 2009, MacDiarmid et al., 1991, Segura-García et al., 2016, Hanson, 2009). The vast majority of scientific evidence of rock lobster diet is based on aquaculture research and captive feeding trials (Mayfield et al., 2001, van Zyl et al., 1998, Butler et al., 2016) or manipulative experiments involving the tethering of prey items, the deployment of infrared cameras adjacent to rock lobster foraging habitat and other similar methods of isolating prey individuals (Pederson and Johnson, 2006, Ling et al., 2009a, Shears and Babcock, 2002).

Currently, there is extensive debate about the spread of invasive marine organisms such as the long spined sea urchin (*Centrostephanus rodgersii*) and the role that rock lobsters play in containing these 'pests'. Considerable research effort on the impact of the sea urchins has been undertaken in Tasmania, supporting the theory that there is considerable spread across a broad geographic range and an increase in urchin density at the sites where they are present and established (Ling and Johnson, 2009, Ling et al., 2009a).

There are many management implications for better understanding the role of rock lobsters within temperate reef communities, most of which revolve around harvest levels and access to fish-able locations. The primary question which has been very difficult to address is the role of rock lobsters in shaping benthic communities (Mayfield and Branch, 2000, Tarr et al., 1996, Hanson et al., 2014). Numerous studies have looked at this question by traditional ecological

sampling techniques- using SCUBA divers and extensive transects to count benthic invertebrates and correlate those potential prey species to rock lobster abundances. These studies are important in gleaning relationships between predators and potential prey species, but there are obvious biases associated with locating these animals in very complex, three dimensional habitats. An issue of great concern is that sea urchins, grazing invertebrates which can exist in cryptic habitats on rocky reefs (Ling et al., 2009a) are a potentially important prey item for rock lobsters and their abundance has been shown to relate to rock lobster population density (Andrew and MacDiarmid, 1991, Haley et al., 2011, Tegner and Levin, 1983a).

At present there are few methods for unravelling the diet of these important crustaceans *in situ* and a paucity of accurate ways to quantify the prey species which they consume. Of particular importance to management of marine ecosystems are the interactions between rock lobsters and other commercially important species, such as abalone (*Haliotis* sp.), and invasive species, such as the long-spined sea urchin (*Centrostephanus rodgersii*).

The rock lobster diet studies that have previously been undertaken primarily employ lethal dissection-based methods which necessitate destroying the lobster (Fielder, 1965, Mayfield et al., 2000a, Haley et al., 2011, Góes and Lins-Oliveira, 2009). A few very early publications reported on lobster diet by dissection, but these studies are quite dated and both the benthos and rock lobster population structure have changed considerably since then (Hickman, 1945).

The use of molecular prey detection techniques has expanded considerably in recent years with a range of predator/prey systems being examined in detail (Agusti et al., 2003a, Blankenship and Yayanos, 2005, Farrell et al., 2000, Jarman et al., 2004, Kasper et al., 2004, Zeale et al., 2011, Clare,

2014). During the Polymerase Chain Reaction (PCR), if prey DNA is present in the dietary 'unknown' sample, this DNA is amplified by paired primers which bind to prey DNA and direct the amplification reaction. PCR is normally carried out over ~40 cycles, with each cycle resulting in the doubling of DNA by amplification. Quantitative Real Time PCR (qPCR) is currently the method of choice for measurement of low concentrations of DNA and there is considerable scope to quantify dietary intake using this technique (King et al., 2008a). Marine mammal diet has recently been examined by using a similar quantitative approach (Deagle and Tollit, 2006, Bowles et al., 2011). By using a suite of species-specific PCR primers and a sensitive quantitative genetics approach (quantitative Real Time PCR) we examine the possibilities of quantifying dietary intake by rock lobsters.

For this experiment, live rock lobsters were kept in captivity and fed specific diets with prey in known ratios. The faecal samples collected after feeding were analyzed by quantitative Real Time PCR (qPCR) to quantify the prey DNA recovered. To assess the molecular prey detection threshold of our current species-specific assays we used captive rock lobsters and fed them on diets of known prey items in a variety of different proportions. After the lobsters were fed, we collected faecal samples and used molecular prey detection assays to quantify the DNA from each prey species in these dietary samples. The prey detection assays employed a quantitative technique, (qPCR) using SyberGreen to determine the amount of prey DNA present in each sample. The RT PCR information was compared with the initial diet proportions from the feeding experiments to determine the sensitivity of our assays. We then used the controlled feeding experimental data to validate the utility of molecular prey detection techniques and to evaluate the quantitative possibilities for these assays for DNA analysis of faecal pellets taken from wild caught rock lobsters. The initial method of controlling rock lobster dietary intake was undertaken by feeding experiments using captive adult rock lobsters and a range of prey species prepared in

various ratios (Table 2). These experiments were followed by an additional set of feeding trials in which rock lobsters were fed material composed of sediments and benthic detritus from sites where urchin barrens were documented in both full and incipient conditions (Table 3).

When using qPCR, multiple dietary 'unknown' samples are run on an instrument concurrently, with each 'unknown' sample occupying a separate tube. In order to generate meaningful quantitative data from each run, it is common practice to also put internal standards into each quantitative PCR experiment. These internal standards are DNA of the target species and they have a known concentration which has been measured by the operator before each reaction begins. A serial dilution of these internal standards is run in order to provide the instrument with DNA of known provenance from a wide range concentrations such as; 1:1 dilution, 1:10 dilution, 1:100 dilution, 1:1000 dilution and 1:10000 dilution.

As the PCR proceeds, the instrument takes a fluorescence reading after each cycle and measures the fluorescence of DNA present in each of the individual 'unknown' samples as well as in each of the internal standards. The fluorescence of the 'unknown' samples is then compared by the instrument to the fluorescence of the internal standards and a calculation is made to determine the relative concentration of the 'unknown' samples in comparison to the internal standard concentrations. Either the raw fluorescence data is used to compare between 'unknown' samples- or the instrument calculations of relative concentrations are used to compare between 'unknown' samples as mentioned above.

The purity and consistency of the internal standard DNA is critical in making comparisons between runs. When extracting DNA from tissues or environmental samples (animals, plants,

soils, etc.) the chemistry to remove and isolate genomic DNA is robust and high concentrations of DNA can be easily purified. Occasionally, however, other smaller molecules can co-precipitate with the DNA and in general these compounds are benign and have little impact on any of the PCR reactions mentioned above. There can be, however, subtle differences in the kinetics of the PCR reaction based upon the amount of these co-purified compounds- particularly when the DNA has been extracted from soils and other mineral rich materials or from animals which have strong chemical defence mechanisms. Very little research has been done on these co-purified compounds as they are generally very small molecules, much smaller than the DNA molecule itself, and beyond the scope and capacity of most molecular genetics facilities to analyse. There can be considerable variation between DNA extractions with regard to these co-purified compounds and one DNA extraction with a given concentration may have considerably more, or considerably less, of these co-purified compounds. So, theoretically, two separate DNA extractions might have the same concentration but they may not amplify by PCR in the same manner due to the variation in co-purified compounds.

4.3 Materials and Methods

Feeding experiments

Rock lobsters for feeding trials were captured by trapping in the Crayfish Point Marine Reserve at Taroona, Tasmania (42.95 °S, 147.34 °E) in March 2007 and April 2010. Lobsters were collected opportunistically ensuring an even distribution of sexes and a wide range of sizes. All captured lobsters were measured and carapace length (CL) was recorded to the nearest millimeter.

Captured lobsters were immediately taken to the laboratory and kept in aerated, flow-through seawater tanks. For the duration of the feeding trials, lobsters were maintained under ambient light conditions and water temperatures in outdoor aquaria at the Institute for Marine and Antarctic Studies Marine Research Laboratories, Taroona, Tasmania.

For each trial the individual lobsters were placed in one section of a 450 L tank separated into three sections with plastic mesh and dividers. Each lobster was provided with a 400 mm x 200 mm concrete block as a shelter. All lobsters were starved for 72 hours prior to each feeding trial to facilitate gut evacuation and to remove any remaining prey DNA from the digestive tract. For each trial, approximately 15 g of food material was given to each lobster as a dietary 'parcel'. This material described in Table 2 was prepared by shucking live common sea urchins (*Heliocidaris erythrogramma*) and long-spined sea urchins (*Centrostephanus rodgersii*) and/or abalone (*Haliotis rubra*) discarding the shells and blending the gonad, muscle and viscera material with a 300W hand mixer (Braun). The sea urchin faecal material was obtained from both urchin species by allowing *H. erythrogramma* and *C. rodgersii* to defecate overnight in aquaria. The blended tissue/faecal material/benthic sediment was mixed with filtered seawater heated to 100° C and gelatin (Davis, New Zealand) was added, stirred, poured into 30ml plastic moulds and allowed to cool.

Benthic sediment samples were collected by SCUBA divers at both locations (Figure 1.); Elephant Rock Research Reserve (ERRR) at St Helens (41.25 °S, 148.35 °E) and the North Bay Research Reserve (NBRR) on the Forestier Peninsula (42.84 °S, 147.92 °E). SCUBA divers used 25 mL HSW sterile syringes (Henke Sass Wolf, GmbH) to obtain benthic sediments (volume approx. 20 ml) at a range of water depths (10 M, 15 M, 20 M and 25 M) and habitats which were associated with

each site. The habitats at Elephant Rock Marine Research Area (ERRR) included both urchin barrens and kelp dominated areas, and the habitats at the North Bay Marine Research Area (NBRR) comprised kelp and incipient sea urchin barrens at a depth of approx. 8 m. Approximately 1 ml of sediment was used for each DNA extraction and for inclusion in the preparation of dietary 'parcels' as described above.

The filtered seawater was heated to 100° C and gelatin (Davis, New Zealand) was added, stirred and poured into 30ml plastic moulds before the gelatin/seawater mixture cooled and set, the component of each diet formula 'parcel' was added to the individual plastic moulds and mixed with a new pipette tip to prevent contamination between diet formulas.

Food 'parcels' were introduced to each lobster at 1700 and individual lobsters were monitored for feeding activity. Only lobsters that actively fed and consumed the entire food sample within the first hour were used in the feeding trials. No additional food was provided to lobsters for the duration of the trial and each lobster was sampled once per feeding trial. Lobsters were selected for faecal collection over the next two days based upon the dietary items given in Table 1 and Table 2. Lobsters were selected randomly to eliminate any tank effect. For each sampling time faecal material was collected from a minimum of three separate lobsters. For each of the individual faecal samples, PCR assays were performed twice to guarantee the consistency of the result and to ensure that there were no pipetting or instrument errors.

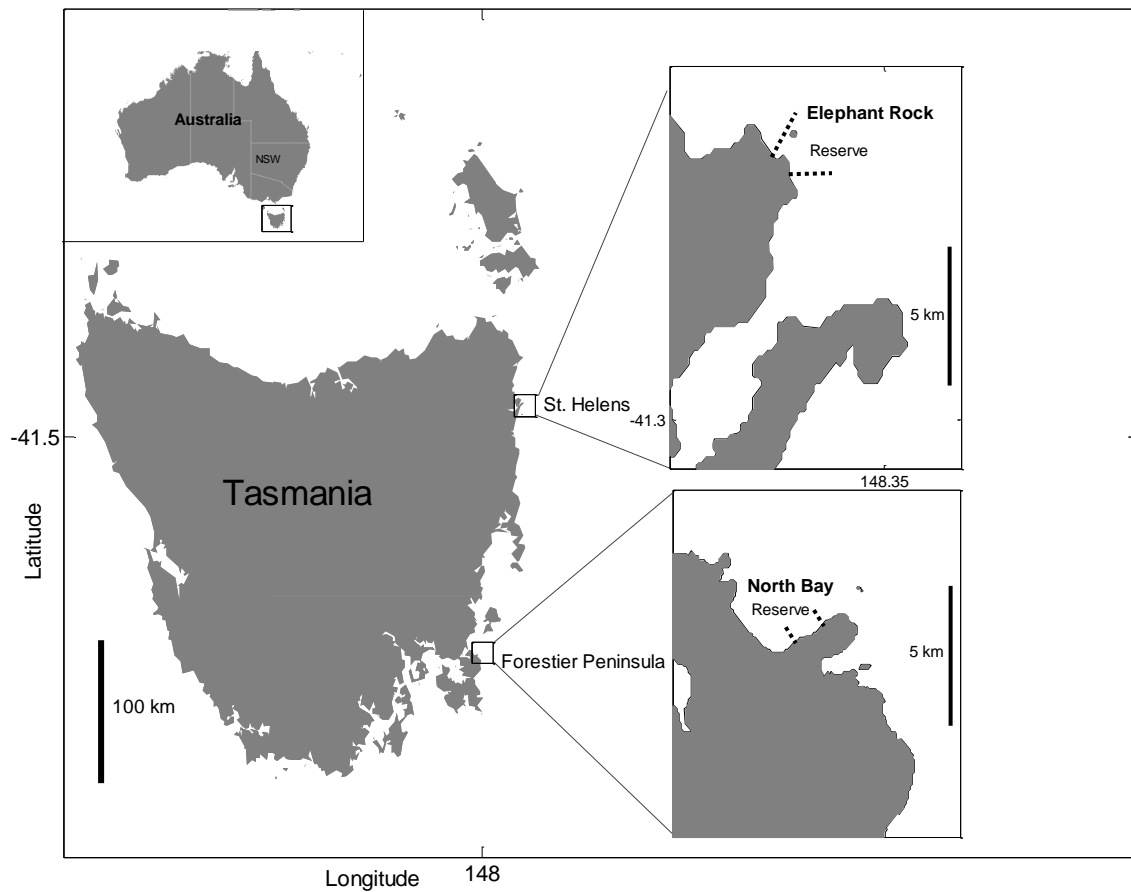


Figure 1. Map showing collection sites in eastern Tasmania where benthic sediment samples listed in Table 2 were collected. Dashed lines on zoomed panels show positions of the research reserve boundaries at Elephant Rock in North Eastern Tasmania (widespread *Centrostephanus rodgersii* sea urchin barrens) and North Bay south eastern Tasmania (incipient *Centrostephanus rodgersii* sea urchin barrens).

Table 1. Feeding trial formulations for quantitative diet experiments using marine invertebrate prey species to validate prey detection by quantitative Real-time PCR (qPCR).

Diet formula	Prey species	Percentage in each diet
AB95_Hel5	<i>Heliocidaris erythrogramma</i>	5%
	<i>Haliotis rubra</i>	95%
AB90_Hel10	<i>Heliocidaris erythrogramma</i>	10%
	<i>Haliotis rubra</i>	90%
AB70_Hel30	<i>Heliocidaris erythrogramma</i>	30%
	<i>Haliotis rubra</i>	70%
AB50_Hel50	<i>Heliocidaris erythrogramma</i>	50%
	<i>Haliotis rubra</i>	50%
AB96_Cent4	<i>Centrostephanus rodgersii</i>	4%
	<i>Haliotis rubra</i>	96%
AB84_Cent16	<i>Centrostephanus rodgersii</i>	16%
	<i>Haliotis rubra</i>	84%
AB50_Cent50	<i>Centrostephanus rodgersii</i>	50%
	<i>Haliotis rubra</i>	50%
AB16_Cent84	<i>Centrostephanus rodgersii</i>	86%
	<i>Haliotis rubra</i>	16%

Table 2. Feeding trial formulations for quantitative diet experiments using benthic environmental samples to validate prey detection by quantitative Real-time PCR (qPCR). Showing sampling location, habitat type and depth for each sample collected.

Sampling location	Habitat type	Depth of collection
Elephant Rock Research Reserve (ERR)	Kelp (K)	10 metres
Elephant Rock Research Reserve (ERR)	Urchin Barrens (UB)	15 metres
Elephant Rock Research Reserve (ERR)	Urchin Barrens (UB)	20 metres
Elephant Rock Research Reserve (ERR)	Urchin Barrens (UB)	25 metres
North Bay Research Reserve (NBRR)	Kelp (K)	10 metres
North Bay Research Reserve (NBRR)	Incipient urchin barrens (IB)	10 metres

Faecal material collection

Lobster faeces were collected using a 100-1000 μ L pipette with disposable tips as described previously (Redd et al., 2008). For each faecal sample a new sterile tip was used to prevent contamination between samples. The tip was inserted directly into the anal pore of the lobster to remove faeces from the hindgut region. The collected material was immediately pipetted into a 1.5 mL micro centrifuge tube containing 500 μ L of MilliQ water and frozen at -20° C as soon as could be arranged, typically within 3 hours of collection. The volume collected varied from approximately 10 μ L to 1 mL depending on the size of lobster and fullness of hindgut at the time of sampling. Water was removed from samples before DNA extraction by centrifuging at 10000 g for 30 s. Excess water was poured off and the sample tubes centrifuged again. Any remaining water was then removed by pipette prior to DNA extraction.

DNA extraction

The Ultra Clean™ Fecal DNA Kit (Mo Bio Laboratories, Inc.) was used for DNA extractions on rock lobster faecal samples following the manufacturer's protocols with the supplied proprietary buffers and reagents. Due to the large number of samples processed in this way, the 96 well format was chosen for time efficiency. All DNA extracted from faecal samples using this kit was ready for PCR and the manufacturer's protocol appeared to remove any potential PCR inhibitors.

Real time PCR amplification

Precautions were taken during preparation of PCR reactions to minimize the possibility of contamination by extraneous DNA. Aerosol-resistant barrier pipette tips were used for preparing all PCR reactions and pipette tips were sterile and pre-packaged. All PCR reactions were set up with a CAS-1200N robotic liquid handling system (Corbett Research) where PCR tubes and pipette tips were subjected to UV light for a minimum of 10 minutes prior to setting up each PCR reaction.

The components of the 14 µL PCRs were as follows: 10 µL SYBRgreen (Sensimix, Quantace, Bioline), 1.25 µL each primer (Geneworks), 1.5 µL 50mM MgCl₂ (Quantace, Bioline), and 2µL (~ 50 ng) template DNA. Both positive and negative controls were run with each batch of PCRs. For negative controls 2 µL MilliQ H₂O was used as template and for positive controls ~ 40 ng template DNA from *C. rodgersii* and *H. erythrogramma* was used to confirm reaction success. For internal standards, plasmids with the 650bp 16s amplicons from *C. rodgersii* and *H. erythrogramma* insertions were used and dilutions of 1:10, 1:100, 1:1,000, 1:10,000 and 1:100,000 were serially

diluted with a CAS-1200N robotic liquid handling system (Corbett Research) to form the basis of standard curves for each qPCR reaction.

The PCR primer sets used in this experiment are listed in Table 3. Primers were obtained from GeneWorks Pty. Ltd. Custom Oligonucleotide service and diluted to 10 μ M for use in setting up PCR reactions. Real-time PCR reactions were set up with a CAS-1200N robotic liquid handling system (Corbett Research) and run for 50 cycles in a RotorGene RG 3000 (Corbett Research) with an annealing temperature at 54 °C.

All samples were analysed twice to exclude the possibility of pipetting or machine errors, ensuring the consistency of results. The first qPCR run was performed using genomic DNA as an internal standard and the second qPCR run was performed using plasmids as internal standards.

Table 3. PCR primers used including sequence of each primer, target organism, and DNA region amplified. DNA regions are mitochondrial nuclear large subunit ribosomal RNA gene (16s rDNA).

Primer Region	Sequence (5'-3')	Target Species	DNA
Centro16sf	GGAACAGCAAACATGGAGAGTCCTGC	<i>Centrostephanus rodgersii</i>	16s rDNA
Centro16sr	CCGTCTTGCCATTCATGCCAGTCTCTA	<i>Centrostephanus rodgersii</i>	16s rDNA
Helio16sf1	TCAAAGGAAGTTACCG	<i>Heliocidaris erythrogramma</i>	16s rDNA
Helio16sr1	CCCTTAAAAGCTTCTGCACCCT	<i>Heliocidaris erythrogramma</i>	16s rDNA
Aba2f	GCTTTTGCTGGAAAACTCG	<i>Haliotis rubra</i>	16s rDNA
Aba2r	CGTCCCCAGTTTATAGGTGA	<i>Haliotis rubra</i>	16s rDNA

4.4 Results:

Rates of detection of sea urchin and abalone DNA in lobster faeces collected during the feeding trials indicate the utility for qPCR detection of these species. The RotorGene RG 3000 (Corbett Research) software package calculates DNA concentration based upon on the Ct value by using the standard curves associated with the DNA standards loaded on each run (Bustin, 2004). To better compare the data between species and runs, we have chosen to simply use the Ct value in all of the results presented. Tables 5 and 6 show the utility of the species-specific primers to amplify DNA directly from environmental samples, and Tables 7 and 8 indicate the detection of prey species DNA in lobsters fed on environmental material.

All samples where qPCR results have a Ct value between 12 and 42 represent detections of the prey species based upon the feeding experiments and the ratios of prey items likely to closely resemble actual predation events by rock lobsters *in situ*. It is important to note that the expected trends in Ct value should be that as concentrations of prey material increase, the value of Ct should decrease- indicating that the PCR amplification crossed the critical threshold earlier due to higher amounts of DNA being present in the reaction. This broadly is the case where the expected amount of sea urchin DNA in the environment decreases with depth and therefore the DNA detections as shown by the increasing Ct value (Table 5). On the other hand, the detection of *H. erythrogramma* increased as the depth from which the samples were taken decreased. This is explained by the ecology of *H. erythrogramma* which is known to occur in greater densities in shallow waters.

The variation in qPCR amplification assay results also suggest that although quantifying dietary intake from wild caught rock lobsters was a primary goal of this experiment, the interpretation of this data remains challenging. The range of detection values (measured as Ct) does not closely match the actual prey ratios fed to the rock lobsters, indicating that variability in digestion and DNA degradation rates will limit the ability to accurately quantify predation activity in field captured rock lobsters. This has also been observed in marine mammals with much larger dietary intakes and faecal volumes (Deagle and Tollit, 2006). It is, however, important to note that the heterogenous nature of marine sediments, the destructive nature of DNA extraction protocols and the consumptive feeding experiments meant that comparisons between particular sediments /feeds do not represent true replication. Specifically, each of the benthic sediment samples collected by SCUBA diving (Table 2) was subsampled to provide three x 1 ml volumes for DNA extraction and ten x 1 ml volumes for feeding trial 'parcels'.

Table 4. Presentation of results from qPCR assays from feeding trials using mixed dietary samples with a list of each dietary formula, percentage of each prey species in the dietary formula and the Ct value (RotorGene RG 3000, Corbett Research) using species-specific PCR primers for each prey item as listed in Table 3.

Dietary formula	Prey species	Percentage in each diet	qPCR result in Ct (mean)
AB95_Hel5	<i>Heliocidaris erythrogramma</i>	5%	34.26
	<i>Haliotis rubra</i>	95%	37.75
AB90_Hel10	<i>Heliocidaris erythrogramma</i>	10%	35.25
	<i>Haliotis rubra</i>	90%	39.34
AB70_Hel30	<i>Heliocidaris erythrogramma</i>	30%	41.69
	<i>Haliotis rubra</i>	70%	40.29
AB50_Hel50	<i>Heliocidaris erythrogramma</i>	50%	37.18
	<i>Haliotis rubra</i>	50%	39.33
AB96_Cent4	<i>Centrostephanus rodgersii</i>	4%	20.49
	<i>Haliotis rubra</i>	96%	41.25
AB84_Cent16	<i>Centrostephanus rodgersii</i>	16%	21.07
	<i>Haliotis rubra</i>	84%	39.31
AB50_Cent50	<i>Centrostephanus rodgersii</i>	50%	22.54
	<i>Haliotis rubra</i>	50%	40.34
AB16_Cent84	<i>Centrostephanus rodgersii</i>	84%	24.24
	<i>Haliotis rubra</i>	16%	38.88

Table 5. Presentation of results from qPCR assays from benthic environmental samples showing habitat type, sampling location and depth for each sample with the Ct value (RotorGene RG 3000, Corbett Research) using species-specific PCR primers for *Centrostephanus rodgersii* (Centro16sf and Centro 16sr).

Sampling location	Habitat type	Depth	qPCR result in Ct (mean)
Elephant Rock Research Reserve (ERRR)	Kelp (K)	10 metres	33.93
Elephant Rock Research Reserve (ERRR)	Urchin Barrens (UB)	15 metres	27.21
Elephant Rock Research Reserve (ERRR)	Urchin Barrens (UB)	20 metres	26.86
Elephant Rock Research Reserve (ERRR)	Urchin Barrens (UB)	25 metres	29.66
North Bay Research Reserve (NBRR)	Kelp (K)	10 metres	No detection
North Bay Research Reserve (NBRR)	Incipient urchin barrens (IB)	10 metres	No detection

Table 6. Presentation of results from qPCR assays benthic environmental samples showing habitat type, sampling location and depth for each sample with the Ct value (RotorGene RG 3000, Corbett Research) using species-specific PCR primers for *Helicoidaris erythrogramma* (Helio16sf1 and Helio16sr1).

Sampling location	Habitat type	Depth	qPCR result in Ct (mean)
Elephant Rock Research Reserve (ERRR)	Kelp (K) n=2	10 metres	33.63
Elephant Rock Research Reserve (ERRR)	Kelp (K) n=3	10 metres	No detection
Elephant Rock Research Reserve (ERRR)	Urchin Barrens (UB)	15 metres	36.12
Elephant Rock Research Reserve (ERRR)	Urchin Barrens (UB)	20 metres	35.26
Elephant Rock Research Reserve (ERRR)	Urchin Barrens (UB)	25 metres	42.07
North Bay Research Reserve (NBRR)	Kelp (K) n=3	10 metres	35.24
North Bay Research Reserve (NBRR)	Kelp (K) n=2	10 metres	No detection
North Bay Research Reserve (NBRR)	Incipient urchin barrens (IB) n=2	10 metres	22.17
North Bay Research Reserve (NBRR)	Incipient urchin barrens (IB) n=3	10 metres	No detection

Table 7. Presentation of results from qPCR assays from feeding trials using benthic environmental samples as feeds with the Ct value (RotorGene RG 3000, Corbett Research) using species-specific PCR primers for *Centrostephanus rodgersii* (Centro16sf and Centro 16sr).

Sampling location	Habitat type	Depth	qPCR result in Ct (mean)
Elephant Rock Research Reserve (ERRR)	Kelp (K)	10 metres	40.1
Elephant Rock Research Reserve (ERRR)	Urchin Barrens (UB)	15 metres	No detection
North Bay Research Reserve (NBRR)	Kelp (K)	10 metres	28.65
North Bay Research Reserve (NBRR)	Incipient urchin barrens (IB)	10 metres	22.91

Table 8. Presentation of results from qPCR assays from feeding trials using benthic environmental samples as feeds with the Ct value (RotorGene RG 3000, Corbett Research) using species-specific PCR primers for *Heliocidaris erythrogramma* (Helio16sf1 and Helio16sr1).

Sampling location	Habitat type	Depth	qPCR result in Ct (mean)
Elephant Rock Research Reserve (ERR)	Kelp (K) n=2	10 metres	No detection
Elephant Rock Research Reserve (ERR)	Urchin Barrens (UB)	15 metres	No detection
North Bay Research Reserve (NBRR)	Kelp (K) n=2	10 metres	No detection
North Bay Research Reserve (NBRR)	Incipient urchin barrens (IB) n=2	10 metres	No detection

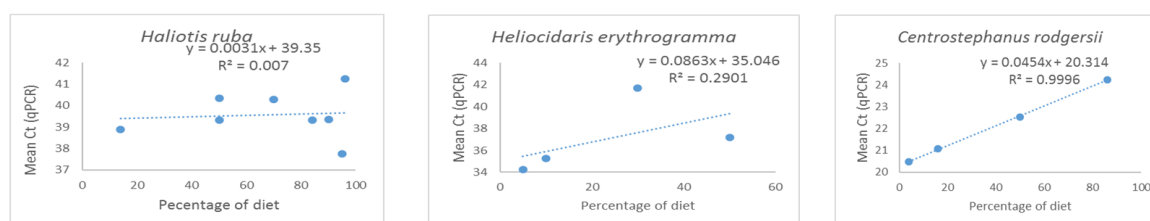


Figure 2. Plots showing correlation between mean Ct value and diet percentages across three dietary items (*Haliotis rubra*, *Heliocidaris erythrogramma* and *Centrostephanus rodgersii*) from mixed dietary samples listed in Table 4. The Ct value (RotorGene RG 3000, Corbett Research) was obtained by using species-specific PCR primers for each prey species.

4.5 Discussion:

The primary benefits of employing molecular techniques to screen diet and examine trophic interactions are the potential to develop non-lethal collection protocols, the unambiguous nature of the assays and the ability to screen for prey species or groups of related prey organisms. Numerous authors have reported on novel methods of obtaining dietary samples such as; collecting faecal material at remote locations (Farrell et al., 2000, Deagle et al., 2009, Dalén et al., 2004), sieving or netting the water adjacent to large marine predators (Jarman and Wilson, 2004, Jarman et al., 2002), and development of specially engineered devices to isolate faecal material (Nejstgaard et al., 2003, Irvin and Tabrett, 2005). Most of these non-lethal collection methods provide material which is of sufficient quality for most molecular analysis techniques. The utility of DNA-based prey detection assays have also been well described with a range of organisms and feeding types shown to be successfully assessed by the sensitive PCR approach (Symondson, 2002, King et al., 2008a).

When interpreting quantitative PCR data, in particular the relative concentration calculations which the Real Time PCR instrument makes, any variation in internal standard DNA can become problematic, and therefore many users of PCR prefer to use a more consistent source of template DNA. This DNA is generated by first creating a fragment of DNA from the target species and then inserting the fragment into a unique circular form of DNA known as a plasmid. Many rapidly growing organisms, such as bacteria, possess these circular plasmids in their cytoplasm and have the ability to transfer them between cells. This is the primary mechanism of passing on traits such as antibiotic resistance between bacteria which mostly reproduce asexually. Bacteria (such as *E.coli*) are easily grown under laboratory conditions, and once a plasmid containing the target DNA fragment has been inserted into a bacterial cell, huge numbers of these cells can be grown

rapidly *in vitro*. Once large colonies of bacterial cells containing the plasmid have been grown, the cells can be 'harvested' and the plasmids isolated. These isolated plasmids are essentially a pure source of the initial target DNA fragment which makes the ideal internal standard for use in quantitative PCR. Because there is virtually no difference in co-purified compounds between plasmids isolated by these procedures, they are more ideally suited to being used for internal standards in quantitative PCR than is genomic DNA.

The use of the qPCR platform does, however, eliminate the need for post-amplification analysis by gel electrophoresis. This not only saves time in the laboratory but also supersedes the scoring of amplicons as present or absent based upon the visual sighting of stained 'bands' on agarose gels. This is a significant improvement for researchers and importantly paves the way for high throughput field experiments using molecular prey detection technologies. The majority of prey detection work which has been published to date has described technological advances and detailed the capacity of DNA-based technologies to different predator/prey systems. The next step forward will be the broadscale use of these techniques to address relevant ecological questions *in situ*. At present, it appears that using the quantitative qPCR approach is best as a very accurate method of ascertaining the presence or absence of prey in predator dietary samples. There are limitations to these molecular techniques and in particular the quantitative interpretations of diet remain extremely challenging (Deagle and Tollit, 2006, McKemey et al., 2003). Although the qPCR has the capacity to precisely measure the amplification progress of the reaction and can deliver immense amounts of data, the biological interpretation of this data remains the issue which is presently complex to resolve. An instance of a dietary sample containing low initial concentrations of prey DNA will illustrate this point more clearly. The qPCR can give a precise quantity of DNA in the sample, yet it is not possible to determine if the predator consumed a very small piece of that prey item in the past few hours, therefore leaving

only a small amount of DNA in the predator gut or if the predator consumed a much larger amount of that prey item perhaps a day ago, and left the same amount of prey item in its gut passage or faecal pellet. The qPCR algorithms can easily and precisely quantify DNA concentrations, but taking that information back to actual predation events and determining the extent, quantity and importance of those predation events remains the large challenge of using this technology to draw greater ecological conclusions. It can also be argued that even the traditional gut content analysis faces the same issues, where the percent of prey species found in a dissected gut are scored in the same manner, and generate the identical questions about recent predation episodes compared to those which took place at a time in the more distant past.

Chapter 5

Using molecular prey detection to quantify rock lobster predation on barrens-forming sea urchins

This chapter has been published as:

Redd, K., Ling, SD., Frusher, SD., Jarman, SD. and Johnson, CJ. (2014). Using molecular prey detection to quantify rock lobster predation on barrens-forming sea urchins. *Molecular Ecology*, 23(15), 3849-3869.

Minor changes to the chapter have been undertaken to ensure consistency in formatting of text, figures and tables

“It goes faster than you think....so watch this space...
‘Cause hands like yours would be a shame to waste ...”

You and Steve McQueen

The Audreys

Between Last Night and Us

2005

Chapter 5: Using molecular prey detection to quantify rock lobster predation on barrens-forming sea urchins

5.1 Abstract

We apply qPCR molecular techniques to detect in situ rates of consumption of sea urchins (*Centrostephanus rodgersii* and *Heliocidaris erythrogramma*) by rock lobsters (*Jasus edwardsii*). A non-lethal method was used to source faecal samples from trap-caught lobsters in winter and summer over two years within two no-take research reserves. There was high variability in the proportion of lobsters with faeces positive for sea urchin DNA, with significant variability across years and seasons dependent on lobster size. Independent estimates of lobster predation rate on sea urchins (determined from observed declines in urchin abundances) suggest that rates of molecular prey detection generally overestimated predation rates. Also, small lobsters known to be incapable of directly predating emergent sea urchins showed relatively high rates of positive tests. These results indicate that some lobsters ingest non-predatory sources of sea urchin DNA, which may include (1) ingestion of *C. rodgersii* DNA from the benthos (urchin DNA is detectable in benthic sediments and some lobsters yield urchin DNA in faeces when fed urchin faeces or sediment); (2) scavenging (small lobsters feed on carcasses of large urchins killed by large lobsters); and (3) predation by rock lobsters on small pre-emergent urchins that live cryptically within the reef matrix (although this possibility could not be assessed). While the DNA-based approach and direct monitoring of urchin populations both indicate high absolute predation rates of large lobsters on emergent urchins, the study shows that in some cases absolute predation rates and inferences of predator-prey interactions cannot be reliably estimated from molecular signals obtained from the faeces of benthic predators. At a broad semi-quantitative level, the approach is useful to identify relative magnitudes of predation and temporal and spatial variability in predation.

Keywords: *Jasus edwardsii*, rock lobsters, sea urchins, *Centrostephanus rodgersii*, *Heliocidaris erythrogramma*, predator-prey interactions, polymerase chain reaction (PCR), quantitative Real Time PCR (qPCR), prey detection, rock lobster diet

5.2 Introduction

Understanding the composition of the diet of individual species is fundamental in defining trophic interactions and relating trophic structure to the functioning of marine communities. Estimating the rate at which particular prey are consumed and thus quantifying overall per capita effects of predators on prey populations is particularly important when interactions involve species capable of exerting overwhelming influence on ecosystem dynamics, such as between kelp grazing sea urchins and their predators on temperate rocky reefs (e.g. sea otters, (Estes and Palmisano, 1974a); clawed lobsters, (Breen and Mann, 1976); fish, (Cowen, 1983); and spiny lobsters, (Tegner and Levin, 1983b, Ling and Johnson, 2009). Marine ecologists have usually identified predator-prey interactions and inferred the effects of consumption either by opportunistic observations (Estes and Palmisano, 1974b, Estes et al., 1998), visual examination of gut contents (Estes et al., 1978, Cowen, 1983) or scats (Estes and Duggins, 1995), by running laboratory trials over days (Tegner and Levin, 1983b, Ling et al., 2009b), conducting trials *in situ* over several months (Ling et al., 2009b, Ling and Johnson, 2012) to tracking abundances of predators and their prey in nature over decades (e.g. reviews by (Babcock et al., 2010a);(Watson and Estes, 2011)). However, given inherent difficulty in directly observing predator-prey interactions, and the possibility of large spatial and temporal variability in these dynamics, determining interaction strengths between species in nature remains a fundamental and challenging task for marine ecologists.

In recent decades, advances in molecular biology have shown that prey DNA recovered from predator faecal material can be used to identify the prey consumed (Symondson, 2002). High resolution molecular tools represent an emerging potential to define and quantify species interactions. As a non-lethal and largely non-intrusive dietary sampling technique, DNA testing of predator faecal material also resolves conservation and ethical issues posed by more traditional approaches to dietary studies that require sacrificing large numbers of animals (Redd et al., 2008,

Jarman and Wilson, 2004). Furthermore, large numbers of samples can be processed quickly and efficiently allowing more quantitatively robust description of food-web structure and thus better inferences of community dynamics. This meets an increasingly urgent need as rapidly changing ocean climates and other human-derived stressors progressively alter marine food webs and lead to major shifts in ecosystem structure and function (Johnson et al., 2011, Wernberg et al., 2011).

On the warming temperate coast of eastern Tasmania (south east Australia), climate-driven range extension of the habitat-modifying sea urchin *Centrostephanus rodgersii* (Diadematidae) poses a considerable ecological threat given this species' capacity to overgraze productive seaweed beds and effect a wholesale shift in reef state and ecology to impoverished sea urchin dominated 'barrens' habitat (Ling et al., 2009a). Owing to grazing by this single species, ~50% of all near-shore rocky reef is maintained as barrens habitat within the sea urchins' native range in New South Wales (Andrew and O'Neill, 2000) and in northeast Tasmania where the urchin first established in Tasmanian waters (Johnson et al., 2005). Thus, the threat of overgrazing in Tasmania is significant, with major implications given that these kelp beds support south east Australia's most valuable fisheries – for southern rock lobster (*Jasus edwardsii*) and black lip abalone (*Haliotis rubra*) – which are not commercially viable on urchin barrens (Johnson et al., 2005). This large ecological shift, associated with ocean warming and range-extension of this habitat-modifying sea urchin, is also influenced by the effects of intensive fishing of the key predator of the sea urchins (Ling and Johnson, 2009, Ling and Johnson, 2012). In eastern Tasmania, field and laboratory experiments show that large rock lobsters (≥ 140 mm carapace length, i.e. 30-35 mm CL above the minimum legal size) are the principal predators of emergent sizes of *C. rodgersii* (i.e. of individuals >70 mm test diameter), but that these large predatory capable lobsters are currently rare due to intense fishing pressure (Ling et al., 2009a).

The impact of fishing on the abundance of large lobsters in eastern Tasmania is demonstrated clearly by long-term monitoring comparing reefs inside marine protected areas with nearby reefs open to intensive fishing (Edgar et al., 2009, Ling and Johnson, 2009). Evidence of cascading trophic effects as a result of rebuilding abundances of large lobsters (*Jasus edwardsii*) within protected areas is evident where native sea urchin species capable of overgrazing in New Zealand (*Evechinus chloriticus*, (Shears and Babcock, 2002)) and Tasmania (*Heliocidaris erythrogramma*, (Johnson et al., 2004) (Pederson and Johnson, 2006, Ling et al., 2010)), occur at relatively low densities in areas where large lobsters are abundant (reviewed by Babcock et al. 2010). In New Zealand, ongoing predator-driven recovery of kelp beds on extensive barrens habitat has been observed to occur over several decades post cessation of fishing (Shears and Babcock, 2003).

In an attempt to determine whether management practices to increase the number of large lobsters would be an efficient means of remediating extensive established *C. rodgersii* barrens, and/or prevent further barrens formation at sites where the urchin is established but barrens formation is at an incipient stage, two no-take research reserves were declared in eastern Tasmania to facilitate rebuilding populations of large lobsters. To accelerate the rebuilding, large lobsters captured in remote areas by the commercial fishery were translocated to each of the research reserves. Here we evaluate the capacity of qPCR molecular techniques to quantify the proportion of sea urchins ingested by large lobsters within these reserves in an attempt to better understand trophic dynamics in this rapidly changing rocky reef system.

5.3 Materials and Methods

Experimental sites

To examine the effects of rebuilding stocks of large lobsters on sea urchin populations and seaweed cover, two no-take research reserves were specifically declared for this purpose in 2008.

Reserve sites were at Elephant Rock in north east Tasmania (hereafter Elephant Rock Research Reserve, ERRR; 41.25°S, 148.35°E; declared 23rd April 2008; an area of extensive overgrazed *Centrostephanus rodgersii* barrens ~200,000 m² in size which constituted ~50% of all nearshore reef at the site), and at North Bay in South East Tasmania where incipient *C. rodgersii* barrens were prevalent (barrens patches ~1-10s m²) (North Bay Research Reserve, NBRR; 42.84°S, 147.92°E; declared 1st November 2008; 'incipient barrens' patches comprised ~1% cover of the total reef area interspersed among otherwise healthy seaweed beds; see Table 1 for further details).

To immediately rebuild a population of large lobsters capable of preying on *Centrostephanus rodgersii* (see Ling et al. 2009b), large lobsters (≥ 140 mm CL) sourced from remote areas by commercial fishers were accumulated in processing facilities and individually tagged before translocation to the research reserves. A total of 1,665 large lobsters (140 – 220 mm carapace length [CL]; total biomass ~2,289 kg) were translocated in this manner. Of this total, 933 large lobsters were translocated to ERRR on two principle release occasions (April and November 2008); while 732 large lobsters were translocated to NBRR over two occasions (May 2009 and March 2010).

Sampling rock lobsters

Faecal samples from individual lobsters were obtained by trapping lobsters within the research reserves during winter and summer seasons over two years post translocation of lobsters (see Figure 2A). Traps were set across the available reef area within the reserves on a regular spaced virtual grid (60 m between grid points). For ERRR, each trap position was assigned to either kelp or sea urchin barrens habitat following intensive video mapping of the benthos at each grid point. As per commercial operations, traps were baited with whole jack mackerel (*Trachurus declivis*)

and cuta (*Thysites atun*) heads, which were deployed on the reef at depths between ~3-45 m. Traps were effective at sampling lobsters to a minimum size of approximately 50 mm carapace length (CL; ~60 g fresh weight) while lobsters below this size, while present at the sites, were likely to escape through the mesh of the trap (25 by 25 mm). Each captured lobster was measured for carapace length to the nearest mm with knife-edge callipers and assigned to size categories of small (≤ 110 mm CL, i.e. undersized lobsters); medium (>110 & <140 mm CL); and large (≥ 140 mm CL), inclusive of large residents and large translocated individuals. Captured lobsters were then sampled for faecal material, tagged (if they were untagged residents), and released at the site of capture.

Faecal material collection

Lobster faeces were collected using a 100-1,000 μL pipette with disposable tips. For each faecal sample a new sterile tip was used to prevent contamination between samples. The tip was inserted directly into the anal pore of the lobster to remove faeces from the hindgut. The collected material was immediately pipetted into a 1.5 mL micro centrifuge tube containing 500 μL of MilliQ water, stored on ice and frozen at -20°C as soon as could be arranged. The volume collected varied from approximately 10 μL to 1 mL depending on the size of lobster and fullness of the hindgut. Rock lobsters which failed to yield a faecal sample were recorded as 'non-feeding' (for proportions of lobster catch deemed to be feeding, refer to Figure 2B). Water was removed from samples before DNA extraction by centrifuging at 10,000 g for 30 s. Excess water was poured off and the sample tubes centrifuged again. Any remaining water was then removed by pipette prior to DNA extraction.

DNA extraction

The Ultra Clean™ Faecal DNA Kit (Mo Bio Laboratories, Inc.) was used for DNA extractions on rock lobster faecal samples following the manufacturer's protocols with the supplied proprietary buffers and reagents. The 96-well format was chosen due to the large number of samples processed and all DNA extracted from faecal samples using this kit was ready for PCR as the manufacturer's protocol appeared to remove any potential PCR inhibitors. DNA was quantified on a NanoDrop 8000 (Thermo Scientific) to determine the total yield in each sample.

PCR amplification

Precautions were taken during preparation of PCR reactions to minimize the possibility of contamination by extraneous DNA. Aerosol-resistant barrier pipette tips were used for preparing all PCR reactions and pipette tips were either sterile and pre-packaged or autoclaved prior to use. All PCR reactions were prepared in a dedicated hood where PCR tubes, pipettes and pipette tips were subjected to UV light for a minimum of 10 minutes prior to setting up each PCR reaction.

The components of the 14 µL PCRs were as follows: 10 µL SYBRgreen (Sensimix, Quantace, Bioline), 1.25 µL each primer (Geneworks), 1.5 µL 50mM MgCl₂ (Quantace, Bioline), and 2µL template which contained approximately 50 ng of total DNA. Both positive and negative controls were run with each batch of PCRs. For negative controls 2 µL MilliQ H₂O was used as template and for positive controls ~ 40 ng template DNA from *Centrostephanus rodgersii* and *Heliocidaris erythrogramma* was used to confirm reaction success. For internal standards, plasmids with the 650bp 16s amplicons from *C. rodgersii* and *H. erythrogramma* insertions were used and serial dilutions of 1:10, 1:100, 1:1,000, 1:10,000 and 1:100,000 obtained using a CAS-1200N robotic

liquid handling system (Corbett Research) provided standard curves for each qPCR reaction. PCR reactions were performed twice to exclude the possibility of pipetting or machine errors.

The PCR primer sets used in this experiment (Table 2) were obtained from GeneWorks Pty. Ltd. Custom Oligonucleotide service, and diluted to 10 μ M for use in setting up PCR reactions. Real-time PCR reactions were set up with a CAS-1200N robotic liquid handling system (Corbett Research) and run in a RotorGene RG 3000 (Corbett Research) for 50 cycles: with an initial 95 °C step for 10 seconds followed by an annealing temperature at 54 °C for 15 seconds followed by an extension step at 72 °C for 20 seconds. At the conclusion of each PCR run, amplicons were confirmed with a melt profile performed at 72 °C – 95 °C rising by 1 °C every 5 seconds.

Filtering PCR amplifications: determining presence of sea urchin DNA

PCR amplification curves were screened for (1) non-normal amplification curves, including a minimum threshold for fluorescence (threshold values of relative fluorescent units (RFU) were set at 0.1 RFU for *C. rodgersii* and 0.6 RFU for *H. erythrogramma* such that curves that appeared otherwise normal but that did not exceed this threshold were considered abnormal and were also removed); and (2) lower and upper cross thresholds (Ct) to minimise effects of false positives as a result of primer dimerization (i.e. reactions that developed unrealistically quickly indicated by Ct values of < 8 cycles; and reactions manifest as normal curves but that took too many cycles to amplify, consistent with primer dimerization, were excluded from consideration; that is, all samples scored as positive had melt curves indicating denaturation of the target amplicon rather than primer dimer, which could be identified as a low t_m peak in negative controls). Thus, positive tests for assays of *C. rodgersii* were considered as those $8 < \text{Ct value} < 40$; and for *H. erythrogramma* as $8 < \text{Ct value} < 45$ (see Appendix 1 for Ct frequency distributions for each urchin

species obtained from analysis of faecal material of trap caught lobsters at the reserve sites field; of lobsters used in aquarium feeding trials – see below; and of sediment samples obtained from the two translocation sites – see Table 5 below).

Recent advances in molecular biology have shown that in some cases prey DNA recovered from predator faecal material can be used not only to identify the prey consumed (Symondson, 2002) but also to quantify the amount ingested (Deagle and Tollit, 2006). Quantification of prey material in dietary samples can be accomplished using genomic DNA standards of known provenance, or to ensure consistency, fragments of amplified DNA are inserted into circular plasmid molecules. These genomic DNA standards or plasmid preparations are then serially diluted and used as internal standard with each PCR experiment. This approach is most appropriate for predator/ prey applications where the assays are directed at particular prey species of interest (Bowles et al. 2011; Deagle & Tollit, 2007).

However for field based samples, quantifying exact or even relative dietary intake is difficult because the amount of prey DNA in faeces is influenced not only by the amount ingested but also by other confounding factors including varying rates of digestion among individuals, the time between ingestion and defecation, the freshness of the recovered faecal material, and the condition of the ingested prey in circumstances where consumption is through scavenging of prey remains. Studies of marine birds and mammals in captivity show that the ratios of prey DNA detected loosely match the ratio of fish species fed during trials (Deagle and Tollit, 2006). But where prior feeding regimes are unknown (such is the case for wild-caught animals) it is currently not feasible to estimate even relative quantities of prey consumed using sensitive molecular techniques, so we adopted binary scoring (0,1). Thus, predation rates of lobsters on sea urchins

were scored as the number of individual lobsters in a given catch testing positive to sea urchin DNA, which we assumed could arise from ingestion of urchin DNA at any time over the previous 3-days (earlier work established that *C. rodgersii* DNA is detectable in lobster faecal samples for 7-60 hours after ingestion, so the assumption of 3 days errs on the conservative; that is, prior feeding trials within controlled laboratory settings confirm that lobsters that have fed within a three day period will have a full hindgut and yield faecal material (Redd et al., 2008).

Analysis of variability in lobster feeding on sea urchins based on assays from field samples

Patterns of variability in the proportion of lobsters positive for sea urchin DNA (as defined by Ct thresholds outlined above) were assessed with binomial Generalised Linear Models (GLMs) with Logit link functions fitted using R (Ver. 2.15.1). GLMs relax the restrictions imposed by standard regression models on both the distribution of the response (here binomial) and the functional relationship between the response and predictors (here logit). Analysis of the deviance of fully-saturated models (including all main and interactive effects), as per standard analysis of variance but substituting classical F-tests for maximum likelihood estimation, were performed separately for each site. For ERRR, a 4-way model was assessed (Year*Season*Size*Habitat) in which there were 2 levels of Year, 2009 vs. 2010; Season, winter vs. summer; Habitat, seaweed bed vs. barren; and 3 levels of Size, small lobsters (≤ 110 mm CL) vs. medium lobsters (>110 & <140 mm CL) vs. large lobsters (≥ 140 mm CL). For NBRR, where habitat consisted entirely of seaweed bed (albeit supporting small incipient barrens patches), the 3-way model consisting of Year*Season*Size was examined.

Potential passive sources of sea urchin DNA: benthic sediments and excreted sea urchin faeces

Direct observations of large rock lobsters during daylight hours indicate that they sometimes appear to 'taste' and / or consume sedimentary material; a feature also noted occasionally for resident individuals (Ling, 2009). It was therefore necessary to assay for the presence of sea urchin DNA in benthic sediments, and to assess the potential for qPCR to detect sea urchin DNA in lobster faeces following ingestion of sediment or cast urchin faeces by rock lobsters. Benthic sediment samples were collected by SCUBA divers at both the ERRR and NBRR sites using 25 mL HSW sterile syringes (Henke Sass Wolf, GmbH) to obtain ~20 ml samples across a range of water depths and habitats. Distinct habitats at ERRR included both sea urchin barrens and adjacent seaweed dominated areas which were sampled at 10 m (seaweed habitat), and 15, 20 and 25 m (barrens habitat) depth, while at NBRR samples were from the seaweed bed and incipient barrens patches within it at a depth of ~8 m.

Feeding lobsters benthic sediment/ sea urchin faecal material

Rock lobsters used in feeding trials were captured by trapping in the Crayfish Point Marine Reserve at Tarooma, Tasmania (42.95 °S, 147.34 °E) in April 2010. Lobsters were collected opportunistically ensuring an even distribution of sexes and a wide range of sizes. The size (carapace length = CL) of all captured lobsters was measured to the nearest mm. Captured lobsters were immediately taken to the laboratory and kept in aerated, flow-through seawater tanks. For the duration of the feeding trials, lobsters were maintained under ambient light conditions and water temperatures in outdoor aquaria at the Institute for Marine and Antarctic Studies Marine Research Laboratories, Tarooma, Tasmania.

For each trial, individual lobsters were placed in one section of a 450 L tank separated into three sections with plastic mesh and dividers. Each lobster was provided with a 400 mm x 200 mm concrete block as a shelter. All lobsters were starved for > 3-days prior to each feeding trial to facilitate gut evacuation and to remove any remaining prey DNA from the digestive tract (Redd et al. 2008). For each trial, fresh sea urchin faecal material was obtained from both species by allowing individuals of *H. erythrogramma* and *C. rodgersii* to defecate overnight in aquaria. To prepare gelatine 'food parcels' based on both the sea urchin faecal material and the benthic sediment samples, filtered seawater was heated to 100° C and mixed with gelatine (Davis, New Zealand), stirred and then poured into 30 ml plastic moulds to which the component of each diet formula (i.e. sediment, or fresh sea urchin faecal pellets, or fresh sea urchin gonad tissue) was added and stirred in once the mixture had cooled to ~25°C using a new pipette tip to prevent contamination between diet formulas. The mix was then allowed to solidify in a standard refrigerator.

A gelatine 'food parcel' (with appropriate dietary element) was introduced to each lobster at 1700 h and individual lobsters were monitored for feeding activity. Only lobsters that fed actively and consumed the entire food sample within the first hour were used in the feeding trials. No additional food was provided to lobsters for the duration of the trial and each lobster was sampled only once in each trial. Lobsters were selected for faecal collection over the next two days at times (hours after commencement of feeding) based upon results of previous experiments to determine the longevity of dietary signals in lobster faeces (Redd et al., 2008). Lobsters were allocated diets randomly to eliminate any possible systematic 'tank' effect. Attempts were made to collect faecal material from at least five replicate lobsters. For each of the individual faecal samples, qPCR assays were performed twice to guarantee the consistency of the result.

Lobster predation rates estimated from decline in sea urchin populations

Independent estimates of lobster predation rates on *C. rodgersii* were obtained by monitoring sea urchin and lobster populations.

Estimating change in sea urchin abundance

Diver-based counts of abundances of emergent sea urchins (*Centrostephanus rodgersii* and *Heliocidaris erythrogramma*) were performed at both the ERRR and NBRR sites using fixed belt-transects (50 m length by 2 m width) to monitor changes in their density. To distinguish changes in sea urchin density that might be attributable to dynamics unrelated to the addition of lobsters and declaration of the reserves, sea urchin densities were also monitored in the same way at nearby control sites (matched by similar reef types, with one to the north and one to the south of each research reserve). For north east sites where rocky reef habitat exists as seaweed bed or widespread sea urchin barrens, a total of 12 independent fixed belt transects were surveyed to assess change in urchin populations within ERRR and at both control sites, with transects established on both seaweed-dominated (n=6) and sea urchin barrens habitats (n=6) at each site (Fig. 1). In the south east, 6 independent fixed belt transects were established within the seaweed bed supporting incipient barrens inside the reserve (NBRR) and outside at both control sites (Fig. 1). In both regions, surveys were conducted on 5 occasions (approximately equally spaced) between 2008 and 2011, with one survey before and four after translocation of large lobsters. To quantify the net change in the populations of both sea urchin species at experimental and control sites in both regions over the entire ~2.5 year study, we compared the first (pre-translocation of lobsters = 'before') and last (= 'after') surveys).

Two approaches were used to assess change in urchin populations at the two experimental sites relative to the appropriate control sites (referred to as C1 and C2 in each region). First we compared the change in urchin density ($= 'B-A' = 'density\ before' - 'density\ after'$, given fixed transects) between control and experimental sites. To minimise risk of Type II error, we first ran a 1-way ANOVA (on the 'B-A' metric) to compare the control sites (C1 and C2 in each region) and assess the possibility of 'post-hoc pooling' of control sites based on the usual criterion $P > 0.25$ in the comparison C1 vs. C2. For *C. rodgersii* in both the NE and SE regions, $P > 0.25$ in the C1 vs. C2 comparison ($P = 0.448$ and 0.284 respectively), so control sites were pooled and compared with the experimental site in each region. For *H. erythrogramma* changes in density at C1 and C2 were similar in the NE ($P = 0.673$) and so control sites were pooled for this region, but in the SE the change in density was different at the two control sites ($P = 0.032$; at one site there was a decline, at the other an increase, in density – see Table 6), so the control sites were not pooled. After pooling (or not), for both urchin species and for both the NE and SE regions, the change in density ('B-A') in the experimental sites and adjacent control sites was compared by 1-way ANOVA.

In the second and complementary approach, which addressed a related but distinctly different null hypothesis, because transects were fixed in space it was possible to separate the independent effects of change in urchin density and spatial variability using paired t-tests to determine whether the change in urchin density ('B-A') at each site differed significantly from zero. In these tests we controlled overall experiment-wise Type I error rates using the Dunn-Sidak adjustment to α for $n = 3$ tests within each region (i.e. reserve and two control sites were examined separately in each region). For the NE, because predatory lobsters were observed to move freely between adjacent habitats (Ling et al. in prep.) and urchins in both habitats were equally accessible to lobsters, benthic transects were pooled across habitats to give an overall

trend of urchin population dynamics at the site level (i.e. $n = 12$ replicate transects for reserve and control sites).

Estimating large lobster abundance

Every translocated and captured resident lobster caught within both ERRR and NBRR was uniquely tagged for individual identification. Trap sampling was performed ~6 monthly at both NBRR and ERRR over the ~2.5 year study, yielding individual “encounter histories” for each lobster (individuals were scored as either, ‘present and alive’ or ‘absent’ at each re-sampling period). This enabled modelling individual survival estimates for translocated (group 1) and resident lobsters (group 2) using the Cormack-Jolly-Seber (CJS) ‘recaptures only’ mark-recapture routine available in the Program MARK® software (White and Burnham, 1999). For CJS, the number of individuals re-sighted alive on subsequent sampling occasions is a function of 2 probabilities: the probability of survival (ϕ), and the probability that a surviving individual is encountered (p). Program MARK® uses Maximum Likelihood estimation to derive estimates of the parameters ϕ and p which maximize the likelihood of witnessing the observed frequency of individuals across different encounter history scenarios.

Following goodness-of-fit testing of the saturated model [i.e. where ϕ and p depend on both lobster group and time, formally denoted ϕ (group*time) and p (group*time)], the most parsimonious CJS model (based on Akaike’s Information Criterion) was then used to inform estimates of the lobster populations using the POPAN routine in MARK®. For translocated lobsters, the estimated apparent ‘survival’ rate (which reflects both survival and emigration of lobsters out of the reserve site) was low immediately post-release of translocated lobsters, as evidenced by the best supported CJS model in which translocated lobsters showed lower survival

than resident lobsters, but thereafter translocated lobsters demonstrated survival rates similar to resident animals. For translocated lobsters, the best estimate of the number retained within the reserve sites was obtained by projecting daily survival rates (obtained by the best supported CJS model) onto the known number of lobsters released over the duration of the study.

Where the starting abundance was unknown, i.e. for resident lobsters, the POPAN model was used to estimate abundances of resident lobsters by size-class (large, 140mm CL; medium ≥ 110 & < 140 mm CL; small, < 110 mm CL) within each reserve at the time of final sampling. The total abundance of large lobsters ≥ 140 mm CL (translocated plus resident lobsters) capable of preying on emergent size-classes of *C. rodgersii* (Ling and Johnson, 2009), and of medium- and large-sized lobsters (translocated plus residents) ≥ 110 mm CL capable of consuming emergent *H. erythrogramma* (Pederson and Johnson, 2006), were estimated for each reserve.

As per the sampling design for diver-based estimates of sea urchin abundances, lobster size and abundance was also estimated in situ by divers (belt transects of 50 by 4 m in dimension were used in NE Tasmania for the ERRR experiment; while geo-referenced searching swims were used to optimally assess lobster abundance in SE Tasmania for the NBRR experiment); with these surveys demonstrating that large predatory size-classes of lobsters are effectively absent/ rare on fished reef outside the research reserve areas (Table 1).

Estimating predation rates

Independent estimates of mortality rates of emergent sea urchins (i.e. excluding the smallest size classes of sea urchins, approx. < 70 mm test diameter, that are restricted to cryptic habitat within the interstices of the reef and not visible or accessible to divers without them rolling boulders)

were determined for comparison with the proportion of sea urchin DNA ingested as obtained from molecular analysis of lobster faecal material. Given consistent and statistically significant declines in sea urchin populations at both reserve sites over the duration of the study (significant declines were observed for both *C. rodgersii* and *H. erythrogramma* at NBRR, and *C. rodgersii* within ERRR; see Table 6), but non-significant changes and lack of consistent direction in the change in urchin abundance at adjacent control sites, we assumed that urchin population declines at the reserve sites were entirely attributable to predation by large lobsters as supported by prior *in situ* demonstrations (Ling and Johnson, 2009) (Ling et al 2009;b; Ling & Johnson 2012). In addition, the net decline of urchins was assessed for the Research Reserves after factoring for change (either incline or decline) in urchin populations at the Control sites. Furthermore, the net increase in lobster abundance within the Research Reserves, i.e. that number of lobsters above and beyond the Control sites, albeit it very low, was also accounted for by calculating the net loss of urchins attributable to the enhanced lobster population within the Research Reserve sites.

For each reserve site and for each species of sea urchin, we estimated the mean number of sea urchins to which each lobster had access, and fitted an exponential decay model based on a three day time step to preserve the observed density of urchins at the beginning and end of the experimental period (observation periods were 955 days at ERRR and 840 days at NBRR). Exponential decay was fitted on the basis of the pattern of mortality observed in four populations of tagged *C. rodgersii* subject to predation by lobsters inside and outside of two marine reserves (Ling and Johnson, 2009) and to patterns of urchin decline at the reserve sites themselves. This is ecologically sensible since it captures declining absolute predation by lobsters as sea urchin densities, and thus encounter rates, decline. We also ran a similar exercise but where the initial and final urchin densities at ERRR and NBRR over the experimental periods were taken as the

mean densities estimated by fitting an exponential decay through all data from every sampling period (note that in this exercise, for NBRR the exponential fit was significantly better than a linear fit, while for ERRR the exponential fit did not provide a better description than a linear fit). Since the estimated predation rates were within 1% across the two methods, here we report on calculations based only on the observed sea urchin densities at the beginning and end of the study inside the research reserves.

Extensive data on movement of individual lobsters provided by VRAP acoustic tagging technology provided robust estimates of the home range area of individual lobsters (Johnson et al., in preparation) and indicated that lobster densities were sufficiently high that home ranges were overlapping at both study sites. On this basis the mean number of sea urchins to which each lobster had access was estimated as the total number of sea urchins in each reserve divided by the number of large lobsters in the reserves. As outlined earlier, based on extensive empirical and experimental observation of size-specific predation on sea urchins by lobsters, large (predatory-capable) lobsters for *Centrostephanus rodgersii* were deemed as those >140 mm CL (Ling and Johnson, 2009) while lobsters >110 mm CL (i.e. medium sized and above) were considered capable of predating *Heliocidaris erythrogramma* (Pederson and Johnson, 2006).

Cross-checking two independent estimates of predation rates

Predation rates derived from DNA detections for each lobster size-class were averaged for all four sampling periods (across seasons and years) to obtain time-integrated average sea urchin predation within the reserves over the study, with mean values and confidence intervals generated from 10,000 bootstrap simulations of the observed variability between different years and seasons. To cross-check DNA based predation estimates within the research reserves, the rate of lobster predation was calculated from the observed decline in urchin abundance using an exponential decay function with 3-day time step from which we calculated the mean number (over the entire study period) of urchins consumed per lobster per 3-day period. Mean values and CIs for 3-day predation rates were estimated from 10,000 bootstrap simulations of the variability in predicted large lobster abundance and variability in the change in urchin abundance across replicate fixed transects surveyed at the start and conclusion of the study within the reserves (to determine net decline of urchins within the Research Reserves relative to Control sites, bootstrap simulations of net change in urchins density for Research Reserves was estimated by factoring this change against the 'Control' dynamics obtained by bootstrapping evenly across both Control sites). Estimating predation rates on urchins based on both the DNA assays and observed declines in urchin densities at the reserve sites assumes that each lobster would not consume more than 1 urchin within any 3-day period. While this assumption may be conservative (deliberately), it is supported by *in situ* remote video surveys of lobsters consuming sea urchins within marine reserves (see Ling et al. 2009a) where, particularly for large urchins, on average no more than a single urchin was observed to be consumed by large individually identifiable lobsters within a 3-day period. In addition, as was the case in deriving overall mean-field estimates of predation rate based on DNA assays pooled across years and seasons, in deriving estimates of predation to explain declines in sea urchins we calculated an average across the entire study period.

5.4 Results

Variability in detection rate of sea urchin DNA in lobster faeces

Detection rates of sea urchin DNA in lobster faeces at ERRR and NBRR indicated ingestion of both *Centrostephanus rodgersii* and *Heliocidaris erythrogramma* tissue across all lobster size-classes examined (Figure 2C). Within the ERRR, which supported extensive *C. rodgersii* barrens, the overall detection rates for *C. rodgersii* and *H. erythrogramma* DNA across all lobster size-classes was similar, at 0.38 and 0.36 respectively. In contrast, within NBRR, the overall rate of detection for *C. rodgersii* DNA in lobster faeces was lower at 0.25, while the detection rate for *H. erythrogramma* was 0.32. These patterns broadly reflected the rank abundance of the two sea urchin species at these sites, i.e. higher *C. rodgersii* and lower *H. erythrogramma* at ERRR, and lower *C. rodgersii* and higher *H. erythrogramma* at NBRR (Table 1). At a finer temporal resolution, detection rates varied significantly across years for *C. rodgersii* and *H. erythrogramma* at both sites variously depending on lobster size and season (Figure 2C; Tables 3,4), and across seasons depending on the year and/or lobster size (Tables 3, 4a) for all but *H. erythrogramma* at NBRR (Table 4b).

In general, the proportion of positive assays to sea urchin DNA increased with increasing lobster size (Figure 2C; and Table 6). Notably, the proportion of smaller lobsters (<140 mm CL) testing positive for sea urchin DNA was higher than expected based on results of in situ field experiments showing that only large lobsters could directly predate sea urchins (Figure 2 and Table 6). For ERRR, the GLM revealed an effect of lobster size on the proportion of positive assays for *Centrostephanus rodgersii* but this was dependent on year and season; lobster size also interacted significantly with season and habitat (Table 3A). For *Heliocidaris erythrogramma* at ERRR, the effect of size was both dependent on habitat and season (Table 3B). Furthermore, comparison of reduced models (stepwise removal of terms) revealed that significantly better fit

was achieved when the habitat factor was included for *H. erythrogramma* (relative to the reduced Year * Season * Size model; $P > \chi^2 = 0.008$), but not for *Centrostephanus* ($P > \chi^2 = 0.246$). At NBRR, lobster size yielded significant effects in combination with year and season for *C. rodgersii*, but lobster size had no effect on *H. erythrogramma* detections at this site where both season and year had significant effects on detection rates for *H. erythrogramma* as main effects (Table 4).

‘Extraneous’ sources and passive ingestion of sea urchin DNA

PCR assays indicated that *Centrostephanus rodgersii* and *Heliocidaris erythrogramma* DNA was present in the unconsolidated sediments accumulated between boulders on the reefs at both sites, and at different depths and habitats (Table 5Ai.). There were also a small number of positive detections of sea urchin DNA in the faeces of lobsters that were fed gelatine ‘food parcels’ containing sediments collected from the benthos at both sites, although on most occasions (10 of 14 tests) no urchin DNA was detected in faeces of lobsters fed sediment, presumably because the digestion process further degraded urchin DNA contained in the sediment (Table 5Aii.). There were also positive detections of sea urchin DNA in the faeces of both *C. rodgersii* and *H. erythrogramma* (obtained from animals collected from incipient barrens patches at NBRR where the species co-occur, Table 5Bi.), and low but non-zero rates of detection of urchin DNA (of both species) in lobster faeces from individuals fed food parcels containing *C. rodgersii* sea urchin faecal pellets (Table 5Bii.). There were no detections of urchin DNA in the faeces of lobsters fed *H. erythrogramma* faeces (Table 5Bii.). We draw particular attention to the result that DNA from one urchin species was sometimes detected in the faecal pellets of the other urchin (suggesting that each species may ingest faecal material of the other in the incipient barrens patches from which the urchins were collected), and that faecal pellets obtained from each species don’t universally contain detectable DNA from that species.

DNA based estimates of predation vs. observed sea urchin declines

Comparing the proportion of lobsters testing positive to sea urchin DNA (averaged across all sampling periods of the study; Table 6i) with estimates determined independently to account for the observed decline in sea urchin abundance at each site (Table 6ii; both proportions represent equivalent instantaneous 3-day ingestion rates averaged over the ~2.5 year study period), revealed broad agreement between methods based on mean values and overlap of 95% CIs. However, for the sizes of lobsters known to prey directly on emergent size-classes of sea urchins, DNA assays consistently showed higher proportions of lobsters testing positive than that required to account for the observed decline in abundance of both sea urchin species at both ERRR and NBRR. Similarly, given prior observations over an extensive range of sizes of both lobsters and urchins showing that only large lobsters are capable of directly preying on emergent sea urchin size-classes for *Centrostephanus rodgersii*, and only medium and large lobsters for the sea urchin *Heliocidaris erythrogramma*, the proportions of positive DNA detections in faecal pellets of smaller size-classes of lobsters were notably high (Table 6, cf. i. & ii.), suggesting ingestion of urchin DNA other than through direct predation.

5.5 Discussion

The potential for molecular detection of predation

The ecological effects of predation on sea urchins by rock lobsters, both on the prey and on broader ecosystem dynamics, continues to be a vexed issue for both management authorities and the rock lobster fishery in Tasmania. Although manipulative experiments have clearly identified the singular importance of rock lobsters as the principle predator of both *Heliocidaris erythrogramma* (Pederson and Johnson, 2006) and *Centrostephanus rodgersii* in this region (Ling et al., 2009a), and the catastrophic impact of overgrazing seaweed beds by sea urchins is all too

apparent (Johnson et al., 2005, Johnson et al., 2012, Ling, 2008, Ling and Johnson, 2012), there remain challenges to providing an unambiguous answer to the question of the magnitude of predation rates on emergent sea urchins across large spatial scales in the field. Only by obtaining such estimates can appropriate 'target' densities of large lobsters be identified so that a sustainable balance between a viable rock lobster fishery and conservation of desired kelp bed habitat can be achieved. The application of molecular prey detection – 'forensic ecology' – can potentially inform these important questions.

Predatory aquatic invertebrates often consume prey with no hard parts (bones, otoliths, scales, etc.) and have extremely efficient digestive systems (Braley et al., 2010, Harper et al., 2005), rendering traditional gut content analysis unreliable or unfeasible, and unlikely to yield quantitative dietary information (Passmore et al., 2006, Read et al., 2006). It is not surprising therefore that despite clear evidence of the importance of rock lobsters as predators of sea urchins (Blamey et al., 2010, Tegner and Levin, 1983a, Mayfield et al., 2001, Shears and Babcock, 2002, Pederson and Johnson, 2006), studies based on analysis of lobster gut contents usually fail to identify sea urchins as prey (Mayfield et al., 2001, Mayfield et al., 2000c, Hickman, 1945). This is possibly due to heavy maceration of urchin hard parts in the gastric mill and/or lobsters only consuming soft tissues such as gonad and connective ligaments through the urchin's peristomial opening while the remainder of the test remains intact and is not consumed (S. D. Ling, pers.obs.).

A further complication is the necessity to sample the population frequently enough to account for the highly seasonal nature of foraging activity (e.g. (Ziegler et al., 2003) and diet structure (Ennis, 1973, Mayfield et al., 2000a) in temperate lobsters. Lobsters in Tasmania present an additional problem because they are the target of a valuable live fishery with a discerning market (Mayfield

et al., 2000c), so to obtain meaningful sample sizes, it is essential that animals are sampled live and returned to the reef at sea or to commercial holding tanks for live trade in perfect condition. The non-lethal faecal collection technique we employed allows for rapid, efficient and repeated sampling with replacement. Thus, for animals such as rock lobsters, DNA-based approaches emerge as a promising tool to assess predation on specific prey across a variety of spatial and temporal scales (Mayfield et al., 2000a, Redd et al., 2008, Chow et al., 2006).

Interpreting molecular detection of prey

Despite the high potential of the technique for the purpose, interpreting molecular detection of prey is far from straightforward, and our work suggests that it may be particularly problematic for benthic foraging species. Where estimates of minimum predation rate are required, it is necessary to consider both the degradation of DNA during digestion in a predator's gut, and the power of PCR to amplify a prey-specific region of DNA from semi-digested material (Deagle et al., 2005b, Nejstgaard et al., 2003, Parsons et al., 2005, Jarman et al., 2002). The longevity of the molecular signal in the lobster *Jasus edwardsii* (from 7-60 hours post consumption, (Redd et al., 2008)) indicates that individuals obtained from traps during routine commercial fishing or research operations can possess prey DNA in their faeces from material consumed prior to the lobster entering the trap. In the commercial lobster fishery in Tasmania traps are typically set for a maximum of 24 hours, so that prey consumed by a lobster within ~30 hours of entering a trap would be detectable using this approach. To be conservative, we assumed that 3 days (72 h) was the maximum time after ingestion that prey could be detected.

It is unlikely that top predators in pelagic environments inadvertently ingest DNA of their usual prey. In contrast, many benthic predators will forage among the detritus and sedimentary

material of the benthos, where they may consume 'extraneous' sources of their prey DNA. Sea urchin DNA is most likely to occur in sediments as a result of release of their faecal material. We found that both sets of sea urchin species-specific PCR primers revealed the presence of sea urchin DNA in most (but not all) samples of total environmental DNA extracted from sediment accumulated between boulders in both incipient and extensive barrens habitat (Table 5). This is not unexpected because marine sediments are well known as repositories for DNA (Bowman and McCuaig, 2003). If lobsters do consume sea urchin DNA by feeding on sedimentary material, then clearly this has the potential to bias estimates of direct predation based on detection of prey DNA in faecal material. The magnitude of the bias will depend on how frequently and how much sediment-associated DNA is consumed, rates of denaturation of prey DNA in the sediment, and the extent of further denaturation of the DNA once it is ingested and passes through the lobster digestive system. It has been previously suggested that rock lobsters may consume marine sediment (Cox et al., 1997, Steyna and Schleyera, 2011), and lobsters (*Jasus edwardsii*) have been reported foraging in sediment-based habitats away from rocky reefs (Langlois et al., 2006). However, the extent to which lobsters ingest sediments and associated detritus is uncertain, in part because direct feeding on sediment is difficult to determine without sacrificing the animal to examine gut contents. We have observed large males active during the day appearing to 'taste' the sediment with their maxillipeds, and while it was not possible to discern from in situ observation whether this sediment or associated detritus is ultimately consumed, it is possible that sedimentary material can be ingested if bound with other food material. While further experimentation is needed to quantify the extent of ingestion of sediment-associated organic material, our initial experiments suggest that prey DNA ingested in this way can lead to positive detection of urchin DNA in lobster faecal material, although detection rates are low suggesting that in most cases ingested DNA is degraded. Even when starved lobsters ingested fresh sea urchin faecal pellets embedded in 'food parcels', in relatively few cases did the lobster faeces subsequently recovered test positive for sea urchin DNA (Table 5). Our tentative conclusion is

that inflation of estimates of direct predation as a result of lobsters ingesting sea urchin DNA from sediments is likely to be low, but nonetheless partially explains the higher than expected rates of DNA detection (particularly for small lobsters) relative to lobster predation rates required to explain observed declines in sea urchin abundance within the research reserves.

There is considerable evidence to suggest that direct predation on adult emergent-sized sea urchins in Tasmania is unlikely other than by large lobsters (Pederson and Johnson, 2006, Ling et al., 2009b, Ling and Johnson, 2012), and there is no case to suggest that lobsters of any size consume other animals that have fed directly on sea urchins. Nonetheless, we cannot discount secondary ingestion occurring as a result of lobsters consuming other prey items that have consumed either urchin faecal material or sediments containing urchin DNA. But more importantly, from extensive deployment of remote infra-red video in situ, we have commonly observed smaller lobsters to scavenge the remains of sea urchins killed by large ones. From 19 'primary kills' of tethered sea urchins by large lobsters at night (that were consumed within the field-of-view), on average we observed an additional 1.47 (SE = 0.22) smaller scavenging lobsters to forage on the fresh sea urchin carcass (i.e. only 40% of all lobsters observed consuming urchins were responsible for the primary kill). Clearly, this is likely to lead to over-estimating direct predation based on detection of DNA in lobster faeces across all lobster size classes, inclusive of large lobsters which were also observed to contest and ultimately scavenge sea urchin kills made by other large lobsters. Notably, this rate of scavenging is, on its own, sufficient to account for all positive DNA detections in small and medium sized lobsters at the ERRR site, although falls short of accounting for all positive detections in small and medium sized lobsters in the incipient barrens at the NBRR (from data in Table 5).

Another possibility to consider is that smaller lobsters incapable of tackling and killing a large emergent sea urchin are able to find and directly predate the smaller sea urchins that live within

the interstices of the reef matrix [and that were not readily available for the predation trials conducted by Ling et al. (2009b)]. This possibility needs to be adequately researched, but we think it is highly unlikely because we have never observed any kill that did not proceed by the lobster standing over the urchin to prise it from the substratum, and then rolling the sea urchin through 180° before penetrating and consuming the soft parts through the soft peristomial region of the urchin's oral surface. In the confines of crevices, using their long spines (which are disproportionately long in juveniles) the urchins wedge themselves into the crevice and it is not possible for lobsters to prise them from the surface to commence the rolling manoeuvre.

It is also possible that estimating predation rates based on DNA analysis of faecal pellets from trap-caught lobsters would underestimate actual predation rates. This would arise if sampling was undertaken during periods when lobsters were not motivated to forage (Ziegler et al. 2002, 2003, 2004), or if the motivation to enter traps baited with fish is lower for lobsters that habitually feed on the urchins (S. D. Ling pers. obs.).

Limitations of qPCR for determining rates of predation for benthic predators

Quantifying dietary intake is arguably the 'holy grail' of study of predator-prey interactions. Recent advances in molecular biology have shown that prey DNA can be used to not only identify the prey being consumed (Symondson, 2002) but also to quantify its intake (Deagle and Tollit, 2006), at least in relative terms. The latter authors showed that ratios of prey DNA in faecal material of marine mammals closely matched the amounts of fish species fed during captive trials (Deagle and Tollit, 2006). However, whether qPCR can be used to quantify either relative or absolute ingestion of prey from field samples is much less certain (Troedsson et al., 2007, Weber and Lundgren, 2009a, Nejstgaard et al., 2008). Because the time of ingestion is unknown for material obtained from wild populations, it is well acknowledged that it is unlikely to be possible

to discern among (i) low levels of ingestion, (ii) high levels of very recent ingestion, or (iii) high levels of ingestion in the relatively distant past, as reasons for observation of relatively low levels of prey DNA in predator faeces.

In addition, our results clearly demonstrate there are arguably even more fundamental considerations in applying and interpreting qPCR to the detection of benthic prey in predator faeces as a means of estimating rates of predation, even when the signal is interpreted at a binary level (i.e. to indicate 'presence' or 'absence' of ingested prey material). This is because there are two elements in the approach that usually require a level of subjectiveness in interpretation, and which can have a bearing on absolute estimates of predation rates. The first is the fluorescence threshold, and the other is the Ct values below or above which amplification curves are deemed not to reflect the presence of target DNA (very low Ct values are usually interpreted as machine error, e.g. commonly ascribed to optics in the qPCR machine, while high values are typically interpreted to indicate primer dimerisation). In the present work, there was an unambiguous discontinuity in the asymptotic value of amplification curves which suggested that identifying the fluorescence 'threshold' and related artefacts in samples was robust. However, decisions on cut-offs for Ct values were not so readily identified. While the few samples with very low Ct scores were well separated from the remaining samples, again suggesting a clear or 'natural' lower limit to identify this kind of erroneous result (see Appendix 1; samples indicating <8 cycles were not interpreted as positive detections), we used the upper limit of Ct values (at 40 cycles for *C. rodgersii* and 45 cycles for *H. erythrogramma*) as the mode of the distribution of Ct scores. While this approach is common, and is defensible at some level, it nonetheless has an arbitrary component, and it needs to be acknowledged that the problem of primer dimerisation as an artefact may begin to arise at Ct values less than or greater than the mode of the distribution of values. Clearly, this decision has a direct effect on results and on estimates of predation rates.

We conclude that it is critical to understand the limitations of qPCR detection of prey items in faeces in addressing trophic interactions and complex ecological questions in general, and for rocky reefs and other benthic systems in particular. The quest to obtain an unambiguous estimate of predation rates by lobsters on emergent sea urchins in the field using qPCR is complicated by the lobsters ingesting sea urchin DNA from sources other than by direct predation, subjective decisions in interpreting qPCR output, and temporal variability in the DNA signal. The latter is likely to reflect real temporal (and spatial) variability, and can be addressed by sampling over several years and seasons to obtain a time-averaged result, as we have done here. The other two issues are not so readily resolved. Thus, while we are encouraged that the two methods used here, at a broad level, give similar results in indicating high rates of predation by lobsters on sea urchins when the prey species occurs at high abundance, sufficient challenges in interpretation remain such that estimates of direct predation based on qPCR cannot be interpreted unambiguously, and are best corroborated by independent approaches. Given this, and significant declines in sea urchin densities at the experimental sites with translocated lobsters, but inconsistent and non-significant changes at control sites, we take the change in urchin density at experimental sites over the ~2.5 years of the study, related to average abundances of large (>140 mm CL) lobsters at these sites over this period, as a robust estimate of absolute predation.

5.6 Acknowledgements

This research was a component of grants: FRDC 2004/013 “Towards integrated multi-species management of Australia’s SE reef fisheries: A Tasmanian example” and FRDC 2007/045 “Rebuilding Ecosystem Resilience-Assessment of management options to minimize formation of ‘barrens’ habitat by the long-spined sea urchin (*Centrostephanus rodgersii*) in Tasmania” which are supported by funding from the Fisheries Research and Development Corporation on behalf of the Australian Government. This project involved a significant collaborative effort from the fishing industry to nominate reefs for use as research reserves and supply of large numbers of lobsters for translocating to these reefs – both of which involved voluntary contributions. We would additionally like to thank all the individuals from IMAS who assisted with rock lobster trapping and/or collection of lobster faecal material at North Bay, Elephant Rock and Crayfish Point, in particular Craig Sanderson, Ruari Colquhoun, Juan Gabriel Dominguez Sarmiento, David ‘Irish’ Faloon, Kylie Cahill, Emma Flukes, Sarah-Jane Pyke, Harrison King and Dan Haley. We thank Simon Wotherspoon for statistical assistance with GLM model construction and bootstrapping to estimate variance. All lobster experiments were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 7th Edition 2004.

5.7 Figures and Tables

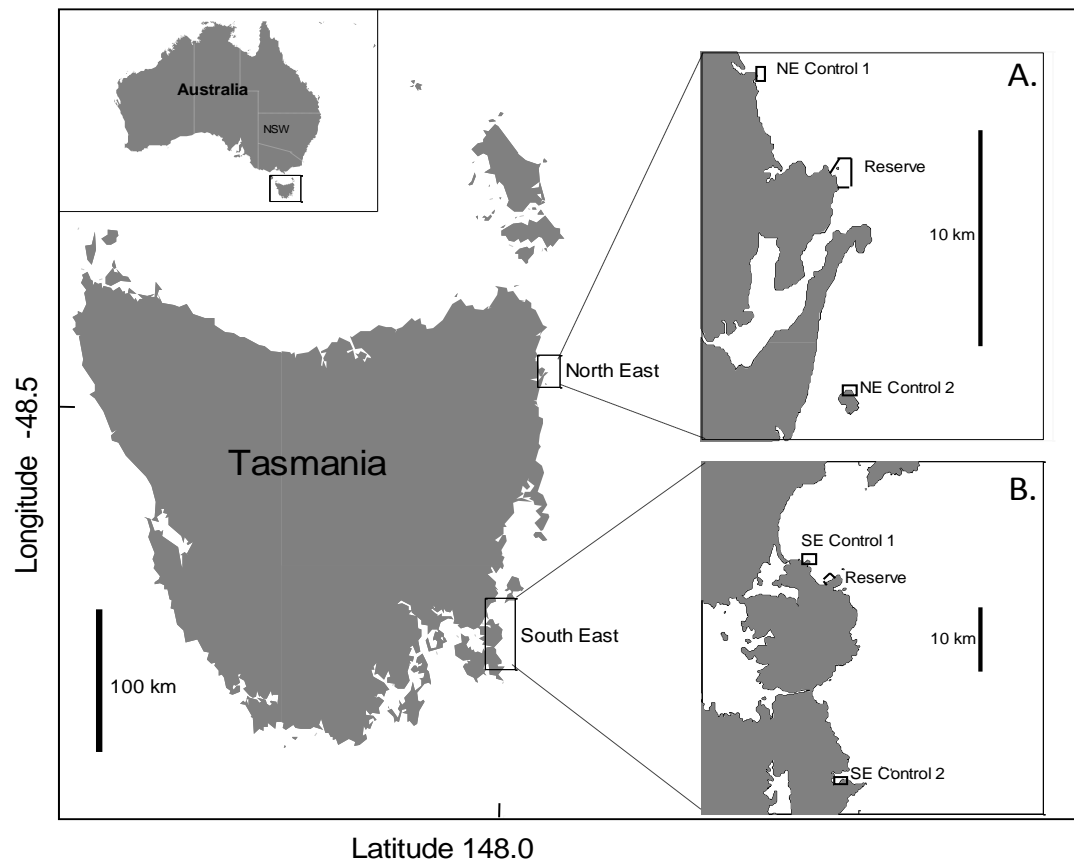


Figure 1. Map showing the research reserves and associated control sites for (A.) Elephant Rock Research Reserve in north eastern Tasmania (widespread *Centrostephanus rodgersii* sea urchin barrens); and for (B.) North Bay Research Reserve in south eastern Tasmania (incipient *C. rodgersii* sea urchin barrens in otherwise intact seaweed beds). The native sea urchin *Heliocidaris erythrogramma* also occurs at all sites.

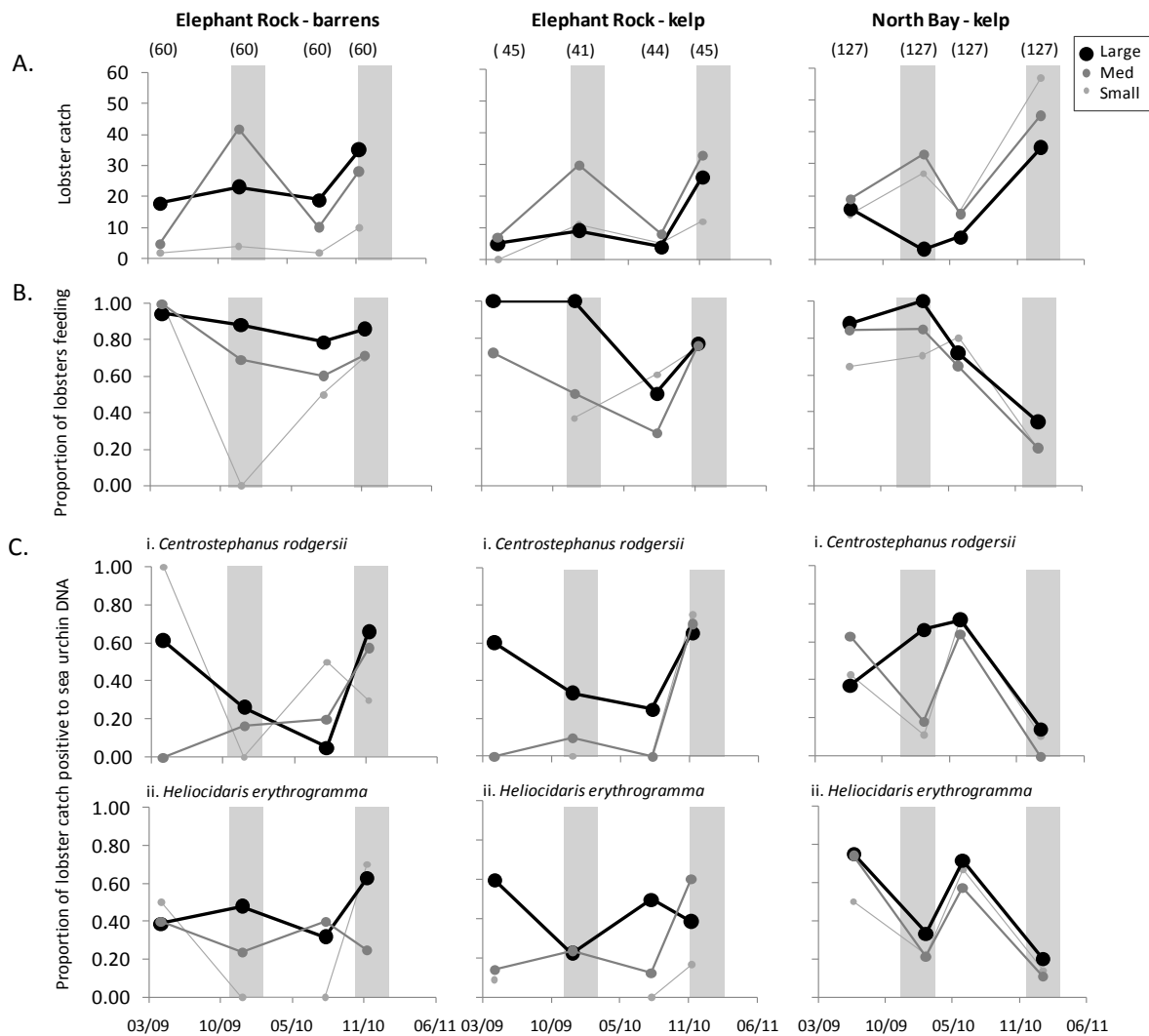


Figure 2. (A.) Catch of lobsters by size-class (see legend) within Elephant Rock and North Bay Research Reserves; number of trap lifts to attain catch is shown in parentheses above each sampling occasion; for large lobsters (which included both translocated and resident lobsters), resident lobsters constituted 8, 13, 12, 25 of the catch across the four sampling periods for ERRR barrens; 1, 8, 2, 17 for ERRR kelp; and 2, 2, 1, 23 for NBRR (B.) Proportion of trap-caught lobsters by size-class deemed to be feeding; i.e. those for which a faecal sample was obtainable. (C.) Proportion of trap-caught lobsters by size-class testing positive to DNA assay for sea urchins (i) *Centrostephanus rodgersii* and (ii) *Heliocidaris erythrogramma* in lobster faecal material sourced from research reserves at Elephant Rock (barrens & seaweed habitats; LHS & middle columns respectively) and North Bay (seaweed / incipient barrens only; RHS column) during winter and summer sampling 2009-2011. Note that lobster size classes are: Large, ≥ 140 mm carapace length (CL); medium, ≥ 110 mm & < 140 mm CL; small, < 110 mm CL. Filled grey regions represent summer periods where feeding rates of lobsters and catch-ability reach an annual high (see Ziegler et al. 2002, 2003, 2004).

Table 1. Habitat distribution and mean abundance of (i) sea urchins (from start to end of the study), and (ii) lobsters retained on reefs inside (A.) Elephant Rock Research Reserve (ERRR) in the NE “Region” of Tasmania; and (B.) North Bay Research Reserve (NBRR) in the SE Region including patterns in abundance at associated Control sites. The ERRR experiment commenced with declaration of the protected area on 21/04/2008, while the NBRR experiment started on 30/09/2008. Note that change in abundance of sea urchin populations and lobster populations were estimated by pooling across both seaweed bed and urchin barrens habitats. Further note that the remaining 17% of reef at ERRR was classified as deep invertebrate community / sediment matrix occurring along the sand edge of the reef at ~35-45 m depth for which we had no diver-based information on sea urchin densities at either the start or end of monitoring. For (ii), lobster abundance estimates are based on mark-recapture ratios of large lobsters CL \geq 140 mm and total legal lobsters CL \geq 110 mm and are averaged over the duration of the study period (contribution of large translocated lobsters to these estimate are shown in parentheses); also shown is the size-specific abundance of lobsters at Research Reserves and Control sites averaged across dive surveys post-declaration of Research Reserves and release of large translocated lobsters to these sites; note that lobster density units are slightly different for each region given that the optimal method of diver-based lobster counts varied between these regions.

Region	Site	Habitat	Area (m ²)	% of reef	(i) Sea urchin population								(ii) Lobster population				
					Density (individ./m ²)				Change in density				Lobster abundance		Lobster density (dive surveys)		
					Start		End		Decline (pooled habitats)		Net decline		Mark-recap. Est. (whole Site)		A. Belt transects (individ. 100/m ²) B. Geo-referenced swims (individ. 100/m ²)		
					C.r	H.e	C.r	H.e	C.r	H.e	C.r	H.e	Large	Legal	Large	Med	Small
A. NE	ERRR	Seaweed beds (>10 m depth)	71 978	16	1.77	0.25	1.31	0.19	0.42	0.08	0.44	0.14	851 (209)	2081	0.156	0.021	0.083
	Control 1				1.89	0.48	1.73	0.35							0.010	0.000	0.010
	Control 2				1.65	0.35	1.49	0.24							0.000	0.031	0.042
	ERRR	Widespread barrens	197 867	43	2.31	0.16	1.92	0.04									
	Control 1				2.24	0.04	2.61	0.02									
B. SE	Control 2				1.59	0.02	1.64	0.03									
	NBRR	Seaweed bed with incipient barrens	175 523	100	0.12	1.26	0.02	0.55	0.10	0.71	0.09	0.64	661 (317)	1713	0.166	0.392	0.978
	Control 1				0.10	1.07	0.05	0.87							0.032	0.150	1.338
	Control 2				0.13	0.72	0.15	0.87							0.006	0.066	0.412

Table 2. (A) PCR primers used including sequence of each primer, target organism or group, and DNA region amplified. DNA regions are mitochondrial nuclear large subunit ribosomal RNA gene (16s rDNA). **(B)** PCR primer testing on primers used, including detailed taxonomy of the species on which primers were tested. Results of primer tests are (+) for positive amplification and (-) for negative amplification of the identified species.

A)

Primer	Sequence (5'-3')	Target Species/Group	DNA Region
Centro16sf	GGAACAGCAAACATGGAGAGTCCTGC	<i>Centrostephanus rodgersii</i>	16s rDNA
Centro16sr	CCGTCTTGCCATTCATGCCAGTCTCTA	<i>Centrostephanus rodgersii</i>	16s rDNA
Helio16sf1	TCAAAGGAAGTTACCG	<i>Heliocidaris erythrogramma</i>	16s rDNA
Helio16sr1	CCCTTAAAAGCTTCTGCACCCT	<i>Heliocidaris erythrogramma</i>	16s rDNA

B)

Phylum	Class	Order	Family	Species	Primers LR-J-12887 LR-N-13398	Primers Helio 16sF Helio 16sR	Primers Aba 2F Aba 2R	Primers Centro 16sF Centro 16sR	Primers CINT 2 F CINT 2 R	Primers Hall16s F Hall16s R	Primers Plag16sF Plag16sR	Primers Pallidus 16sF Pallidus 16sR	Primers Maorum16s F Maorum16s R	Primers Jas16sF Jas1sR
Crustacea	Malacostraca	Decapoda	Palinuridae	<i>Jasus edwardsii</i>	+	-	-	-	-	-	-	-	-	+
Crustacea	Malacostraca	Decapoda	Plagusidae	<i>Plagusia chabrus</i>	+	-	-	-	-	-	+	-	-	-
Crustacea	Malacostraca	Decapoda	Portunidae	<i>Nectocarcinus integrifrons</i>	+	-	-	-	-	-	-	-	-	-
Mollusca	Cephalopoda	Octopoda	Octopodidae	<i>Octopus macrurus</i>	+	-	-	-	-	-	-	-	+	-
Mollusca	Cephalopoda	Octopoda	Octopodidae	<i>Octopus pallidus</i>	+	-	-	-	-	-	-	+	-	-
Mollusca	Gastropoda	Orthogastropoda	Helicidae	<i>Helix rubra</i>	+	-	+	-	-	+	-	-	-	-
Mollusca	Gastropoda	Patellogastropoda	Acmaeidae	<i>Notoacmaea petterdi</i>	+	-	-	-	-	-	-	-	-	-
Mollusca	Gastropoda	Caenogastropoda	Muricidae	<i>Thais orbita</i>	+	-	-	-	-	-	-	-	-	-
Mollusca	Gastropoda	Archaeogastropoda	Trochidae	<i>Austrocochlea constricta</i>	+	-	-	-	-	-	-	-	-	-
Mollusca	Gastropoda	Archaeogastropoda	Turbinidae	<i>Turbo undulatus</i>	+	-	-	-	-	-	-	-	-	-
Mollusca	Gastropoda	Littorinomorpha	Littorinidae	<i>Afulittorina praeterrisica</i>	+	-	-	-	-	-	-	-	-	-
Echinodermata	Echinoida	Echinoida	Echinometridae	<i>Heliocidaris erythrogramma</i>	+	+	-	-	-	-	-	-	-	-
Echinodermata	Echinoida	Diadematoidea	Diademidae	<i>Centrostephanus rodgersii</i>	+	-	-	+	+	-	-	-	-	-

Table 3. Analysis of deviance for binomial GLM model fitted to presence/ absence of detections of sea urchin DNA in lobster faecal material for (A.) *Centrostephanus rodgersii*; and (B.) *Heliocidaris erythrogramma* at Elephant Rock Research Reserve 2009-2010. Signif. codes: '***' <0.001; '**' <0.01; '*' <0.05.

A.	Source	Df	Deviance	Resid. Df	Resid. Dev	P(> Chi)	
	NULL			347	460.96		
	Year	1	28.69	346	432.27	8.5E-08	***
	Season	1	9.58	345	422.69	0.002	**
	Size	2	9.07	343	413.62	0.011	*
	Habitat	1	0.24	342	413.39	0.628	
	Year*Season	1	42.41	341	370.98	7.4E-11	***
	Year*Size	2	6.36	339	364.62	0.042	*
	Season*Size	2	6.84	337	357.78	0.033	*
	Year*Habitat	1	1.57	336	356.2	0.210	
	Season*Habitat	1	1.57	335	354.64	0.210	
	Size*Habitat	2	0.77	333	353.87	0.682	
	Year*Season*Size	2	10.23	331	343.64	0.006	**
	Year*Season*Habitat	1	1.21	330	342.42	0.271	
	Year*Size*Habitat	2	0.69	328	341.73	0.708	
	Season*Size*Habitat	2	7.63	326	334.1	0.022	*
	Year*Season*Size*Habitat	1	0.00	325	334.1	1.000	

B.	Source	Df	Deviance	Resid. Df	Resid. Dev	P(> Chi)	
	NULL			347	455.6		
	Year	1	6.70	346	448.9	0.010	**
	Season	1	1.07	345	447.83	0.301	
	Size	2	9.95	343	437.88	0.007	**
	Habitat	1	0.84	342	437.04	0.359	
	Year*Season	1	5.69	341	431.36	0.017	*
	Year*Size	2	1.14	339	430.21	0.565	
	Season*Size	2	0.30	337	429.91	0.861	
	Year*Habitat	1	0.05	336	429.86	0.820	
	Season*Habitat	1	0.05	335	429.82	0.828	
	Size*Habitat	2	9.26	333	420.55	0.010	**
	Year*Season*Size	2	3.09	331	417.47	0.214	
	Year*Season*Habitat	1	0.87	330	416.6	0.352	
	Year*Size*Habitat	2	5.38	328	411.22	0.068	
	Season*Size*Habitat	2	9.80	326	401.42	0.007	**
	Year*Season*Size*Habitat	1	0.28	325	401.14	0.598	

Table 4. Analysis of deviance for binomial GLM model fitted to presence/ absence data of positive DNA tests of lobster faecal material for (A.) *Centrostephanus rodgersii*; and (B.) *Heliocidaris erythrogramma* at North Bay Research Reserve 2009-2010. Signif. codes: '***' <0.001; '**' <0.01; '*' <0.05.

A.	Source	Df	Deviance	Resid. Df	Resid. Dev	P(> Chi)	
	NULL			284	319.98		
	Year	1	3.91	283	316.07	0.048	*
	Season	1	61.63	282	254.44	4.1E-15	***
	Size	2	0.06	280	254.39	0.973	
	Year*Season	1	7.87	279	246.52	0.005	**
	Year*Size	2	6.45	277	240.07	0.040	*
	Season*Size	2	6.53	275	233.55	0.038	*
	Year*Season*Size	2	3.21	273	230.34	0.201	

B.	Source	Df	Deviance	Resid. Df	Resid. Dev	P(> Chi)	
	NULL			284	355.48		
	Year	1	9.11	283	346.38	0.003	**
	Season	1	56.38	282	289.99	6.0E-14	***
	Size	2	1.69	280	288.31	0.431	
	Year*Season	1	0.78	279	287.53	0.376	
	Year*Size	2	1.15	277	286.38	0.563	
	Season*Size	2	0.50	275	285.88	0.779	
	Year*Season*Size	2	0.79	273	285.09	0.673	

Table 5. (A.) Detections of sea urchin DNA in (i.) benthic sediment and (ii.) faecal samples taken lobsters fed in the laboratory with ‘food parcels’ containing sediments from both research reserves. (B.) Detections of sea urchin DNA in (i.) sea urchin faecal material, (ii.) in lobster faecal material for lobsters fed with ‘food parcels’ containing urchin faecal material and (iii) fresh urchin gonad. Numbers in parentheses indicate the number of independent replicate samples. Note that due to mixed success in obtaining faecal material from lobsters fed artificial ‘food parcels’ under laboratory conditions (a minimum of 5 attempts were made per treatment), replicate faecal samples from these lobsters were variable and generally low.

<i>Samples detecting positive for the presence of sea urchin DNA</i>							
A.	Site	Habitat	Depth	i). Sediment		ii). Lobsters fed sediment	
				<i>C.r</i>	<i>H.e</i>	<i>C.r</i>	<i>H.e</i>
ERRR		Kelp	10 m	80% (5)	20% (5)	0% (2)	0% (2)
		Barrens	15 m	100% (5)	60% (5)	50% (2)	50% (2)
		Barrens	20 m	100% (5)	80% (5)	<i>no data</i>	<i>no data</i>
		Barrens	25 m	100% (5)	60% (5)	<i>no data</i>	<i>no data</i>
NBRR		Kelp	10 m	100% (5)	60% (5)	0% (2)	0% (2)
		Incip. barrens	10 m	100% (5)	40% (5)	0% (1)	0% (1)

B.	Sea urchin material source	i). Sea urchin faeces		ii). Lobsters fed sea urchin faeces		iii) Lobsters fed sea urchin gonad	
		<i>C.r</i>	<i>H.e</i>	<i>C.r</i>	<i>H.e</i>	<i>C.r</i>	<i>H.e</i>
	<i>Centrostephanus rodgersii</i>	75% (4)	50% (4)	27% (11)	55% (11)	64%(14)	10%(10)
	<i>Heliocidaris erythrogramma</i>	75% (4)	50% (4)	0% (1)	0% (1)	13%(8)	44%(9)

Chapter 6

Utility of 454 sequencing technology in unravelling predator/prey relationships inside and outside marine protected areas

“...So well we once were the jesters
in your Kingdom by the sea
and now we’re out to be the masters
for to set our spirits free ...”

Janglin’

Edward Sharpe and the Magnetic Zeros

Up From Below

2009

Chapter 6: Utility of 454 sequencing technology in unravelling predator/prey relationships inside and outside marine protected areas

6.1 Abstract

Dietary samples of the southern rock lobster (*Jasus edwardsii*) were analysed using clone library and pyrosequencing/ 454 sequencing approaches with the goal of better understanding the role of the rock lobster on temperate reefs. A quick and non-lethal method was used to collect rock lobster dietary samples from both Marine Protected Areas (where lobsters are protected from fishing) as well as sites where rock lobster fishing pressure is high. Although only a small number of individuals were analysed in this pilot scale study, the DNA dietary results provided valuable insights into the breadth of diet including feedback mechanisms for species which had previously only been considered as lobster predators, and confirmation of feeding on non-reef associated species. Differences between fished and non-fished sites provided insights into the diversity of prey species in addition to supporting broader observations on changes in the abundance of species within these regions. These results highlight the potential for 454 sequencing to be a valuable tool for understanding trophic linkages in marine systems.

Key Words *Jasus edwardsii*, rock lobsters, prey detection, rock lobster diet, pyrosequencing, 454 sequencing, clone library, prey inventory, ecological impacts of fishing, climate change

6.2 Introduction

The challenge of accurately describing the entire dietary inventory of a predator rests upon the ability to compile an exhaustive list of both frequently consumed items as well as all of the uncommon species which play a more minor role in the diet. All predator/prey interactions are relevant from an ecological perspective and therefore the complete and accurate prey inventory is an extremely worthwhile goal to attain. Of particular importance are species which are important or key components of ecosystems and that are being altered through non-natural processes such as exploitation or anthropogenic climate change.

Rock lobsters are Australia's most valuable fishery (Norman-López et al., 2014) and the southern rock lobster, (*Jasus edwardsii*) is a dominant predator on coastal reefs in south eastern Australia. The market value of this species is high for live animals and, although managed by a quota and size limits, the exploitable portion of the population is heavily harvested. Although considered as a key component of temperate reef ecosystems in SE Australia and New Zealand (Edgar and Barrett, 1999, Shears and Babcock, 2002) and in other marine environments (Lord and Dalvano, 2015, Haarr and Rochette, 2012, Hagen and Mann, 1992, Perez-Barros et al., 2010, Follesa et al., 2009), defining the diet of lobsters has been problematic (Mayfield et al., 2000a). The traditional lethal gut content analysis sampling strategy has the potential to encounter unidentifiable material which has been mechanically or chemically degraded by the predator during the digestion process (Hyslop, 1980). The ability to accurately decipher this type of complex and degraded material is one of the main strengths of the laboratory-based methods of dietary analysis such as ELISA, protein chemistry and DNA.

DNA is a relatively stable molecule comprised of a sequence of nucleotides which can survive many degrading inputs, making DNA ideally suited to dietary forensics. At a molecular level, the partially digested prey material can be sufficiently robust to provide DNA for downstream analysis such as amplification by polymerase chain reaction (PCR) (Casper et al., 2007a), cloning using bacterial plasmids and vectors (Deagle et al., 2005a) and, more recently, 454/pyrosequencing (Deagle et al., 2010, Brown et al., 2012). The analysis of any dietary samples containing degraded DNA has numerous parallels with other forensic applications as the constraints on the DNA templates are similar (Symondson, 2002). In theory, any complex sample with mixed DNA templates can have individual sequences separated by molecular methods and those sequences can be accurately identified (Dorigo et al., 2002, Bowman and McCuaig, 2003).

For the broad screening approach outlined in this chapter, samples were collected from animals in the wild with a completely unknown diet necessitating a 'library'/inventory approach to screen prey diversity using both a clone library and a 454/pyrosequencing platform. These techniques are well known from microbial ecology and have been used extensively to characterize and describe bacterial communities (Bowman and McCuaig, 2003, Brown and Bowman, 2001, Kelly and Chistoserdov, 2001) and have occasionally been used to screen prey diversity (Deagle et al., 2005a, Tiede et al., 2016). In the clone library approach, the dietary sample DNA is first amplified by PCR using universal primers. These primers are designed to amplify DNA from all eukaryotic organisms (Kasper et al., 2004) and the resulting PCR products are then ligated into plasmids and inserted into host *E.coli* bacterial cells to construct a clone library of the full sample diversity (Hughes et al., 2001, Bowman and McCuaig, 2003). The host bacterial cells are then grown on agar plates and harvested after reaching a sufficient size. The resulting bacterial plasmids are finally isolated and sequenced and the sequences compared to those in the National Center for Biotechnology Information (GenBank) nucleotide database using the BLAST search function and the Barcode of Life (BOL) using the Identify Specimen utility.

Pyrosequencing technology is a relatively new DNA sequencing method based on a principle of sequencing by synthesis (Gharizadeh et al., 2006). This DNA sequencing technique begins with a template strand of DNA and employs four enzymatic reactions to synthesize the complementary DNA strand (Diggle and Clarke, 2004). As the DNA synthesis proceeds, each of the enzymatic reactions incorporates nucleotides, producing a sequence peak signal, and much like the qPCR platform, the reactions are bioluminometric and tracked in real time. Theoretically every single strand of DNA in the sample is sequenced individually and therefore the entire DNA composition of the sample is known, generating a large volume of individual DNA sequence data. This is particularly useful for the situation where mixed DNA templates are present, as in the case of environmental samples, complex microbial communities (Roesch et al., 2007, Callaway et al., 2010) and dietary samples (Deagle et al., 2009, Deagle et al., 2010, Pegard et al., 2009). Due to

the powerful nature of pyrosequencing and the demand for the application, the technology has rapidly become more accessible and the data processing considerably more efficient. The method has proven highly suitable for the detection of single nucleotide polymorphism analysis (Silvar et al., 2011) and sequencing of short stretches of DNA, which make it ideal for use in molecular prey detection assays. Although the pyrosequencing procedure is relatively straightforward, the experimental design and primer development are important considerations when considering a pyrosequencing 'run'.

To minimize the risks associated with using a new technology and to determine the suitability of the method for rock lobsters, the design of the rock lobster prey detection pyrosequencing experiment outlined in this chapter was relatively simple. Several rock lobster dietary samples were selected from animals caught across a range of locations including the Maria Island Marine Protected Area (MIMPA), a reserve site considered to be un-fished, and several locations within the same region which are subjected to considerable commercial and recreational fishing pressure. The population density and sizes of rock lobsters at these fished locations varies considerably with the MIMPA (Edgar and Barrett, 1999, Barrett et al., 2003). The legal commercial and recreational size limit for male and female rock lobsters in Tasmania is 110 mm and 105 mm carapace length (CL) respectively and at the fished locations very few animals were found at this size or greater. At the MIMPA, by contrast, large rock lobsters (greater than 140mm CL) dominate the catch with relatively few lobsters at or below the legal size. In this chapter I provide insights into the merit of DNA dietary analysis to be used to understand trophic linkages and to demonstrate the potential to develop relatively comprehensive lists of dietary items for lobsters. In doing so, this chapter demonstrates the proof of concept of DNA dietary studies to provide substantial new data in the role played by rock lobsters on temperate reefs, the ecosystem level impacts of fishing, and the shifts in diet due to climate change and other environmental factors.

6.3 Materials and Methods

Sample collection

Specimens of the southern rock lobster (*Jasus edwardsii*) were collected from several locations around Tasmania including the Maria Island Marine Protected Area (MIMPA) at (42.58 °S, 148.06 °E), the North Bay Research Reserve (42.84 °S, 147.92 °E) and around the fishing grounds of Tasman Island (43.24 °S, 148.02 °E), Bicheno (41.87 °S, 148.30 °E), and Recherche Bay (43.55 °S, 146.89 °E), Tasmania. At all locations, lobsters were captured using traps baited with jack mackerel (*Trachurus declivis*)/ Pacific mackerel (*Scomber australasicus*) and coua (*Thyrsites atun*) heads which were deployed on rocky reef bottom or, occasionally, on sand immediately adjacent to rocky reef habitat in depths of ca. 3-70 m. Lobsters were also collected at Recherche Bay SCUBA diving ensuring an even distribution of genders and a wide range of sizes.

Lobster faecal samples used in this experiment were collected from the 'non-fished' locations at the MIMPA in 2005 and 2006 and at North Bay Marine Protected Area in 2010. At 'fished' locations (Tasman Island, Bicheno and Recherche Bay) lobster faecal samples were collected in August 2005.

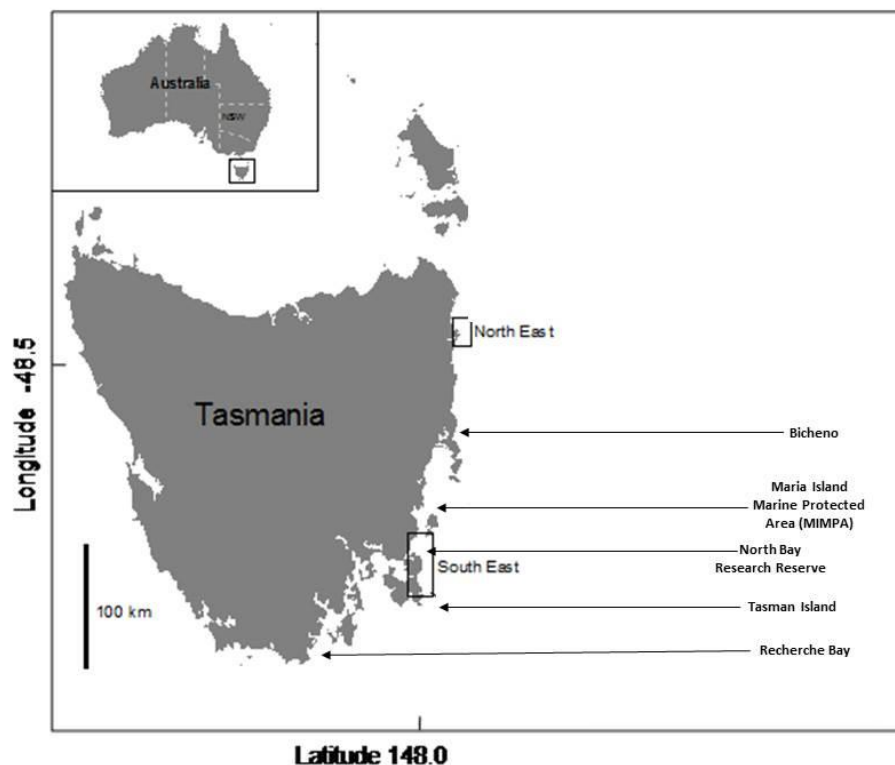


Figure 1. Map showing collection sites in eastern Tasmania where rock lobster dietary samples described were collected. Collection sites at Bicheno, Tasman Island and Recherche Bay are considered heavily fished locations and the collection sites at Maria Island Marine Protected Area and North Bay Research Reserve are classed as unfished locations.

Lobster faeces were collected using a Pipetteman™ 100-1000 µL pipette with disposable tips as described previously (Redd et al., 2008). For each faecal sample a new sterile tip was used to prevent contamination between samples. The tip was inserted directly into the anal pore of the lobster to remove faeces from the hindgut region. The collected material was immediately pipetted into a 1.5 mL micro centrifuge tube containing 500 µL of 70% ethanol. Ethanol has been shown to be an effective preservative for field samples and does not require freezing or any special handling (Jarman et al., 2004). The volume collected varied from approximately 10 µL to over 1 mL depending on the size of lobster and fullness of hindgut at the time of sampling. Faecal samples were collected and stored in 1.5 mL micro centrifuge tubes with 500 µL 70% ethanol.

Ethanol was removed before DNA extraction by centrifuging at 10000 g for 30 s. Excess ethanol was poured off and the sample tubes centrifuged again.

DNA extraction

The Ultra Clean™ Fecal DNA Kit (Mo Bio Laboratories, Inc.) was used to perform DNA extractions on all rock lobster faecal samples following the manufacturer's protocols with the supplied proprietary buffers and reagents. All DNA extracted using this kit was ready for PCR and the manufacturer's protocol appeared to remove any potential contaminants and PCR inhibitors.

PCR Amplifications

For cloning, DNA was amplified using universal primers which target a region of the 16s rRNA (Kasper et al., 2004). PCR thermal cycling conditions for LRN and LRJ primers (Table 1) were: denaturation and DNA polymerase activation at 95 °C for 10 min followed by 10 initial amplification cycles: 94 °C for 30 s, annealing at 52 °C for 30 s, 72 °C for 1 min of the 35 cycles. The final extension step was 72 °C for 5 min and the reaction was held at 12 °C until removed from the MJ Research PTC-2001 Thermal Cycler.

Prior to cloning, PCR products were visualized by electrophoresis on 1.5% agarose gels stained with Gel Red™ (Biotium, Hayward, California) with 15 µL Gel Red™ per 100 mL agarose. Each gel was loaded with 5 µL of PCR product and 2 µL of loading dye. A 100 bp ladder was used on every gel to determine fragment size and to confirm the results of each PCR. All agarose gels ran for 20 min at 120 V.

Table 1. Universal PCR primers used for cloning library including sequence of each primer, target organism or group, and DNA region amplified. DNA regions are mitochondrial nuclear large subunit ribosomal RNA gene (16s rDNA).

Primer	Sequence (5'-3')	Target Species/Group	DNA Region
LRN-13398	CGC CTG TTT ATC AAA AAC AT	Universal	16s rDNA
LRJ-12887	CCG GTC TGA ACT CAG ATC ACG T	Universal	16s rDNA

Clone library

PCR products from rock lobster faecal samples were cloned into vector pCR[®] 4-TOPO[®] and used to transform One Shot[®] TOP 10 Chemically Competent *Escherichia coli* bacterial cells following the manufacturer's instructions. *E. coli* cells were spread onto LB agar plates prepared with 20 µL of X-gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside) and 50 µL of transformed bacteria according to manufacturer's instructions. After 24 h of culture of LB agar plates at 37 °C, transformed colonies/clones were selected and picked with a 10 µL pipette tip and put into 2 ml of sterile LB medium in 10 ml tubes. These were incubated overnight at 37 °C on a shaking incubator (Bioline) and the bacteria were centrifuged and any remaining LB media was removed.

Plasmids were isolated with the Ultra Clean[™] 6 Minute Mini Plasmid Prep Kit (Mo Bio Laboratories, Inc.) and stored in 10 mM Tris prior to sequencing. All plasmid sequencing was conducted with an Applied Biosystems 3730XL DNA Analyzer under BigDye[™] Terminator cycling conditions.

DNA sequences were initially viewed with Sequencher™ and all plasmid vector sequences were removed with this program. Sequences were then compared to those in the National Center for Biotechnology Information (GenBank) nucleotide database using the BLAST search function and the Barcode of Life Datasystems (BOLD), using the Identify Specimen utility.

454/Pyrosequencing trials

For 454/pyrosequencing, DNA was amplified using Multiplex Identifier (MID)-tagged universal primers (Table 2) which were designed to target a region of the 16s rRNA of marine invertebrates with the exclusion of the predator (*Jasus edwardsii*). Real-time PCR reactions were run for 50 cycles in a RotorGene RG 3000 (Corbett Research) with an annealing temperature at 54 °C. The components of the 14 µL PCRs were as follows: 10 µL Bioline SensiFAST SYBR No-Rox mix (Quantace, Bioline), 1.25 µL each primer (Macrogen), 1.5 µL 50mM MgCl₂ (Quantace, Bioline), and 2µL (~ 50 ng) template DNA. The PCR primer sets used in this experiment are listed in Table 2 and all MID-tagged primers were obtained from Macrogen Custom Oligonucleotide service (Macrogen) in a dry condition and were diluted to 10 µM for use in setting up PCR reactions.

All (MID) amplified DNA fragments were quantified on a Nanodrop 8000 at 260 nm to prepare the samples for the GS FLX Titanium 454 sequencing platform. All equimolar, amplified DNA fragment samples were then pooled and sequenced uni-directionally using the GS FLX Titanium chemistry on a Genome Sequencer FLX Instrument (Roche).

Approximately 2000-3000 sequence reads were captured from each sample listed in Table 3 and each set of sequences was initially quality checked to remove noise as described in (Quince et al., 2011, Huse et al., 2007) and (Quince et al., 2009). Sequences were then converted to FASTA

format and the primer and MID tags removed before being clustered into operational taxonomic units (OTUs). Each OTU was identified to the nearest possible prey species by individual Basic Local Alignment Search Tool (BLAST) searches of the NCBI GenBank Nucleotide database (Blaxter et al., 2005). All OTU identifications were made when matches were greater than 90% to the sequence in the GenBank Nucleotide database. Occasionally there were several species which matched the OTU query, and then the species which was known to occur in the local area was chosen over species from other ocean basins/continents (Blaxter et al., 2005).

Table 2. Multiplex Identifier (MID)-tagged universal primers used including sequence of each primer. DNA regions are mitochondrial nuclear large subunit ribosomal RNA gene (16s rDNA).

Primer	Adaptor Sequence (5'-3')	MID (5'-3')	Primer (5'-3')
Uni_16S1F_1	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACGAGTGCGT	GGACGAGAAGACCCT
Uni_16S2R_1	CCTATCCCCTGTGTGCCTTGGCAGTCTCAG	ACGAGTGCGT	CGCTGTTATCCCTATGGTAACT
Uni_16S1F_2	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACGCTCGACA	GGACGAGAAGACCCT
Uni_16S2R_2	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACGCTCGACA	CGCTGTTATCCCTATGGTAACT
Uni_16S1F_6	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATATCGCGAG	GGACGAGAAGACCCT
Uni_16S1R_6	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATATCGCGAG	CGCTGTTATCCCTATGGTAACT
Uni_16S1F_7	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CGTGTCTCTA	GGACGAGAAGACCCT
Uni_16S1R_7	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CGTGTCTCTA	CGCTGTTATCCCTATGGTAACT
Uni_16S1F_8	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTCGCGTGTC	GGACGAGAAGACCCT
Uni_16S1R_8	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTCGCGTGTC	CGCTGTTATCCCTATGGTAACT
Uni_16S1F_10	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TGATACGTCT	GGACGAGAAGACCCT
Uni_16S1R_10	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TGATACGTCT	CGCTGTTATCCCTATGGTAACT
Uni_16S1F_11	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CATAGTAGTG	GGACGAGAAGACCCT
Uni_16S1R_11	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CATAGTAGTG	CGCTGTTATCCCTATGGTAACT

Table 3. Capture locations and fishing status/condition of each location, showing sizes and sexes of rock lobsters sampled and dates of sampling for dietary analysis and prey diversity screening by molecular methods experiment. Small sized lobsters range from 85mm CL to 109mm CL, and large sized lobsters range from 110mm CL to 200mm CL.

Capture location	Fishing status	Size of lobsters		Sex of lobsters	Capture Date	Method
Maria Island	Marine Protected Area	Large	n=2	Female	June 2006	Trap
Maria Island	Marine Protected Area	Large	n=3	Male	June 2006	Trap
Maria Island	Marine Protected Area	Large	n=3	Male	April 2006	Trap
Recherche Bay	Fished location	Small	n=3	Male/Female	September 2005	Dive
Maria Island	Marine Protected Area	Large	n=3	Female	April 2006	Trap
Tasman Island	Fished location	Small	n=5	Male/Female	August 2005	Trap
North Bay	Marine Protected Area	Large	n=4	Male/Female	June 2010	Trap

6.4 Results

Clone libraries were generated from faecal samples from 5 rock lobsters captured at a fished location (adjacent to Tasman Island) in SE Tasmania. The combined sample yielded 5 OTUs (Table 4). These OTUs corresponded to a range of prey species and 11.8% of the clones sequenced contained DNA from rock lobster (*Jasus edwardsii*).

All (MID) amplified DNA fragments which were sequenced on the GS FLX Titanium 454 sequencing platform clustered into 340 OTUs. These OTUs corresponded to a broad range of marine invertebrate and chordate prey species as listed in Tables 5-11.

Table 4. Clone library results from rock lobster (*Jasus edwardsii*) dietary samples collected at the fished location around Tasman Island, Tasmania in August 2005. All data presented is based upon PCR amplifications of the mitochondrial nuclear large subunit ribosomal RNA gene (16s rDNA) and molecular cloning of separate amplicons of mixed DNA dietary sample templates and includes the percentage of the clone library which was attributed to each of the species as well as the percent match for that sequence using the NCBI Blast Nucleotide utility.

Prey species	Percentage of clones	Sequence match
<i>Octopus maorum</i>	73.5%	100%
<i>Jasus edwardsii</i>	11.8%	100%
<i>Dromia dehaani</i>	5.9%	94%
<i>Florometra serratissima</i> (Crinoidea)	5.9%	86%
Uncultured archaeon (Archaea)	2.9%	91%

Table 5. Pyrosequencing library results from rock lobster (*Jasus edwardsii*) dietary samples collected from two large female rock lobsters captured at Maria Island Marine Protected Area during winter 2006. Showing composition of prey in these dietary samples by relative number of sequences in each sample (%) and the best taxonomic resolution of each prey item (>97% sequence match in GenBank).

Common name	Group	Species	Percentage
Limpet	Mollusc	<i>Montfortula rugosa</i>	0.38%
Red algae	Seaweed	<i>Rhodophyta sp.</i>	0.77%
Bacterial pathogen	Bacteria	<i>Spiroplasma sp.</i>	3.08%
Bait	Fish	<i>Scomber australasicus</i>	15.77%
Sponge	Sponge	<i>Callyspongia fallax</i>	32.88%
Bait	Fish	<i>Thyrsites atun</i>	47.12%

Table 6. Pyrosequencing library results from rock lobster (*Jasus edwardsii*) dietary samples collected from three large male rock lobsters captured at Maria Island Marine Protected Area during winter 2006. Showing composition of prey in these dietary samples by relative number of sequences in each sample (%) and the best taxonomic resolution of each prey item (>97% sequence match in GenBank).

Common name	Group	Species	Percentage
Marine diatom	Diatom	<i>Navicula sp</i>	0.12%
Marine worm	Arenicolidae	<i>Abarenicola affinis</i>	0.24%
Octopus	Octopus	<i>Adelieledone piatkowski</i>	0.24%
Comb-finned squid	Squid	<i>Chtenopteryx sicula</i>	0.36%
Marine worm	Microphallidae	<i>Maritrema eroliae</i>	0.61%
Bait	Fish	<i>Thyrsites atun</i>	0.61%
Ciliate	Protozoa	<i>Tetrahymena pyriformis</i>	0.73%
Squid	Squid	<i>Sepia sp.</i>	0.73%
Bait	Fish	<i>Scomber australasicus</i>	0.73%
Maori octopus	Octopus	<i>Octopus maorum</i>	0.97%
Octopus	Octopus	<i>Octopus sp.</i>	1.70%
Little weed whiting	Fish	<i>Neoodax balteatus</i>	1.94%
Wheel shell	Snail	<i>Zethalia zelandica</i>	2.18%
Marine worm	Steganodermatidae	<i>Lepidophyllum steenstrupi</i>	3.64%
Red algae	Seaweed	<i>Rhodophyta</i>	4.12%
Common urchin	Urchin	<i>Heliocidaris erythrogramma</i>	13.33%
Top shell snail	Snail	<i>Prothalotia lehmanni</i>	29.58%
Top snail	Snail	<i>Family Trochidae</i>	38.18%

Table 7. Pyrosequencing library results from rock lobster (*Jasus edwardsii*) dietary samples collected from three large male rock lobsters captured at Maria Island Marine Protected Area during autumn 2006. Showing composition of prey in these dietary samples by relative number of sequences in each sample (%) and the best taxonomic resolution of each prey item (>97% sequence match in GenBank).

Common name	Group	Species	Percentage
Scorpion fish	Fish	<i>Helicolenus sp.</i>	0.08%
Red algae	Seaweed	<i>Rhodophyta sp.</i>	0.23%
Heart urchin	Urchin	<i>Echinocardium cordatum</i>	0.23%
Conger eel	Fish	<i>Conger verreauxi</i>	0.38%
Sponge	Sponge	<i>Aplysina fulva (Oscarella sp.)</i>	0.41%
Maori octopus	Octopus	<i>Octopus maorum</i>	1.55%
Purple wrasse	Fish	<i>Notolabrus fucicola</i>	2.41%
Bait	Fish	<i>Thyrsites atun</i>	2.49%
Scarlet wrasse	Fish	<i>Pseudolabrus miles</i>	2.53%
Tooth brush leatherjacket	Fish	<i>Acanthaluteres vittiger</i>	2.56%
Swell shark	Shark	Family: <i>Scyliorhinidae</i>	4.64%
Bait	Fish	<i>Trachurus declivis</i>	8.93%
Little weed whiting	Fish	<i>Neodax balteatus</i>	73.58%

Table 8. Pyrosequencing library results from rock lobster (*Jasus edwardsii*) dietary samples collected from three small rock lobsters captured at fished locations in Recherche Bay during spring 2005. Showing composition of prey in these dietary samples by relative number of sequences in each sample (%) and the best taxonomic resolution of each prey item (>97% sequence match in GenBank).

Common name	Group	Species	Percentage
Feather star	Crinoidea	<i>Antedon sp</i>	0.05%
Brittle star	Ophiuridae	<i>Ophiuridae sp</i>	0.05%
Robust small mouth	Fish	<i>Nansenia ardesiaca</i>	0.10%
Strange Pathogen	Bacteria	<i>Lagenidium sp</i>	0.10%
Checkered top shell	Snail	<i>Austrocochlea odontis</i>	0.15%
Purple Wrasse	Fish	<i>Notolabrus fucicola</i>	0.45%
Blue throated wrasse	Fish	<i>Notolabrus tetricus</i>	0.65%
Bait	Fish	<i>Trachurus declivis</i>	0.95%
Common urchin	Urchin	<i>Heliocidaris erythrogramma</i>	1.95%
Inflated urchin	Urchin	<i>Holopneustes inflatus</i>	9.29%
Maori octopus	Octopus	<i>Octopus maorum</i>	9.99%
Sponge	Sponge	<i>Sponge</i>	76.27%

Table 9. Pyrosequencing library results from rock lobster (*Jasus edwardsii*) dietary samples collected from three large female rock lobsters captured at Maria Island Marine Protected Area during autumn 2006. Showing composition of prey in these dietary samples by relative number of sequences in each sample (%) and the best taxonomic resolution of each prey item (>97% sequence match in GenBank).

Common name	Group	Species	Percentage
Maori octopus	Octopus	<i>Octopus maorum</i>	0.05%
Marine diatom	Diatom	<i>Cylindrotheca fusiformis</i>	0.09%
Blue throated wrasse	Fish	<i>Notolabrus tetricus</i>	0.09%
Purple Wrasse	Fish	<i>Notolabrus fucicola</i>	0.11%
Seastar	Starfish	<i>Meridiastra oriens</i>	0.14%
11 armed seastar	Starfish	<i>Coscinasterias muricata</i>	0.27%
Common triplefin	Fish	<i>Forsterygion lapillum</i>	0.55%
Heart urchin	Urchin	<i>Echinocardium cordatum</i>	1.07%
Leatherjacket	Fish	<i>Acanthaluteres vittiger</i>	1.25%
Sponge	Sponge	<i>Aplysina fulva</i> (<i>Oscarella</i> sp.)	1.68%
Conger Eel	Fish	<i>Conger verreauxi</i>	2.82%
Swell Shark	Shark	Family: <i>Scyliorhinidae</i>	4.73%
Bait	Fish	<i>Sardinops sagax</i>	7.49%
Bait	Fish	<i>Trachurus declivis</i>	17.61%
Little weed whiting	Fish	<i>Neoodax balteatus</i>	27.94%
Bait	Fish	<i>Thyrsites atun</i>	34.11%

Table 10. Pyrosequencing library results from rock lobster (*Jasus edwardsii*) dietary samples collected from five small rock lobsters captured at fished locations around Tasman Island during winter 2005. Showing composition of prey in these dietary samples by relative number of sequences in each sample (%) and the best taxonomic resolution of each prey item (>97% sequence match in GenBank).

Common name	Group	Species	Percentage
Bearded rock cod	Fish	<i>Pseudophycis barbata</i>	0.27%
Bait	Fish	<i>Thyrsites atun</i>	0.38%
Swell Shark	Shark	Family: <i>Scyliorhinidae</i>	0.43%
Scorpion fish	Fish	<i>Helicolenus</i> sp.	0.59%
Red algae	Seaweed	<i>Rhodophyta</i>	1.34%
Purple Wrasse	Fish	<i>Notolabrus fucicola</i>	6.51%
Maori octopus	Octopus	<i>Octopus maorum</i>	90.48%

Table 11. Pyrosequencing library results from rock lobster (*Jasus edwardsii*) dietary samples collected from four large rock lobsters captured at a temporary marine research reserve (declared 24 months prior to the sample being taken) at North Bay during winter 2010. Showing composition of prey in these dietary samples by relative number of sequences in each sample (%) and the best taxonomic resolution of each prey item (>97% sequence match in GenBank).

Common name	Group	Species	Percentage
Horseshoe leatherjacket	Fish	<i>Meuschenia hippocrepis</i>	0.11%
Swell shark	Shark	Family: <i>Scyliorhinidae</i>	0.38%
Conger eel	Fish	<i>Conger verreauxi</i>	0.54%
Red algae	Seaweed	<i>Rhodophyta sp.</i>	0.70%
Sponge	Sponge	<i>Agelas schmidtii</i>	2.05%
Little weed whiting	Fish	<i>Neoodax balteatus</i>	5.44%
Spiny-tailed leatherjacket	Fish	<i>Acanthaluteres brownii</i>	5.93%
Bait	Fish	<i>Trachurus declivis</i>	9.48%
Bait	Fish	<i>Thyrsites atun</i>	12.66%
Butterfly perch	Fish	<i>Caesioperca lepidoptera</i>	16.86%
Common urchin	Urchin	<i>Heliocidaris erythrogramma</i>	45.85%

The large number of species and the broad taxonomic categories supports the claims that rock lobsters are opportunistic scavengers. As the dietary composition within a particular lobster, or a small group of lobsters within a site, often reflects this opportunism, the occurrence of prey items across different sites and different time periods provides stronger insights into dietary preferences. As the taxonomic resolution of algae and sponges was low, these two taxonomic groups have been removed from this summary although it is noteworthy that they both occurred in 5 of the 7 groups analysed. Of the 35 species recorded, 25 (71%) were recorded only once. The most frequently occurring species - little weed whiting (*Neoodax balteatus*), swell shark (Family: *Scyliorhinidae* but most likely *Cephalocylium laticeps*), purple wrasse (*Notolabrus fucicola*) and Maori octopus (*Octopus maorum*) occurred on 4 occasions and the conger eel (*Conger verreauxi*) and common urchin (*Heliocidaris erythrogramma*) occurred on 3 occasions.

Although low in sample sizes and reflecting different sizes of lobsters there are a number of interesting observations. Lobsters in the MIMPA tended to have a broader diet than in fished regions and cephalopods and molluscs tended to be more consistent in the diets of lobsters in the reserve. While echinoderms and teleosts occurred in approximately similar numbers in both fished and protected sites, the species encountered were different with only one in seven urchin species and five of thirteen fish species being common to fished and unfished regions (Table 12).

Table 12. Comparison of the total number of species found in lobsters sampled in the Maria Island Marine Protected Area (MIMPA; n = 11) and in Fished locations (n=12). The taxonomic resolution for algae and sponge was low and thus these groups have been aggregated to one per group.

Group	Total	MIMPA	Fished	Percentage Common
Algae	1	1	1	100%
Annelids	3	3	0	0%
Cephalopods	5	5	1	20%
Chondrichthyans	1	1	1	100%
Echinoderms	7	4	4	14%
Molluscs	5	4	1	20%
Sponge	1	1	1	100%
Teleosts	13	8	10	38%
Total	36	27	19	

Table 13. Comparison of the total number of species found in the diet of rock lobsters sampled across all locations. Marine Protected Areas include Maria Island, established more than ten years at the time of sampling and North Bay, established less than two years at the time of sample. Fished locations include Tasman Island and Recherche.

Species	Maria Island <i>Females</i> (Autumn 2006)	Maria Island <i>Males</i> (Autumn 2006)	Maria Island <i>Males</i> (Winter 2006)	Maria Island <i>Females</i> (Winter 2006)	North Bay (Winter 2010)	Tasman Island (Winter 2005)	Recherche (Spring 2005)
Common urchin			13.33%		45.85%		1.95%
Heart urchin	1.07%	0.23%					
Inflated urchin							9.29%
Seastars	0.41%						
Feather star							0.05%
Brittle star							0.05%
Maori octopus	0.05%	1.55%	0.97%			90.48%	9.99%
Octopus			1.94%				
Wheel shell			2.18%				
Top shell snail			29.58%				0.15%
Top snail			38.18%				
Limpet				0.38%			
Squid			1.09%				
Sponge	1.68%	0.41%		32.88%	2.05%		76.27%
Red algae		0.23%	4.12%	0.77%	0.70%	1.34%	
Marine diatom	0.09%		0.12%				
Marine worms			4.49%				
Bacteria				3.08%			
Swell shark	4.73%	4.64%			0.38%	0.43%	
Conger eel	2.82%	0.38%			0.54%		
Leatherjackets	1.25%	2.56%			6.04%		
Whittings	27.94%	73.58%	1.94%		5.44%		
Wrasse	0.20%	4.94%				6.51%	1.10%
Butterfly perch fish					16.86%		
Scorpion fish		0.08%				0.59%	
Triplefin fish	0.55%						
Bearded rock cod						0.27%	
Bait species	59.21%	11.42%	1.34%	62.89%	22.14%	0.38%	0.95%

Differences are also apparent in the percentage contribution to the diet in both fished and unfished areas (Figure 1 and Table 13). Although a greater variety of cephalopods were found in the faecal material of lobsters in the MIMPA, cephalopods made a much greater contribution to the diet in fished regions. The species of echinoderms that occurred in the faecal material in the fished regions also made a greater contribution to the diet. In the MIMPA, the broader variety of molluscs also reflected a higher contribution to the diet. Teleosts also made a greater contribution to the diet of lobsters in the MIMPA. Algae, annelids and chondrichthyans were a minor source of diet in both groups and sponges were a consistent high portion of the diet across both groups.

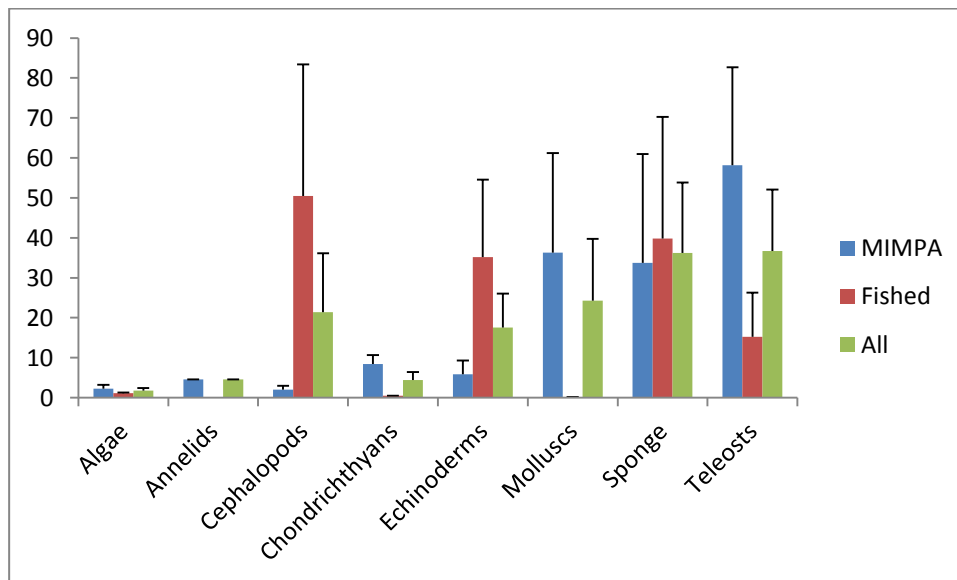


Figure 1: Mean percentage contribution to the diet of the major groups in MIMPA and Fished sites. Error bars are standard errors.

6.5 Discussion

Dietary studies on top predators are key in unravelling their role in the ecosystem, and the emergence of DNA-based approaches opens the door to a more broad study of trophic relationships, particularly on animals with high conservation value or in areas where lethal sampling is unsuitable such as in Marine Protected Areas (Casper et al., 2007b, Sheppard and Harwood, 2005). Both the clone library and the 454 pyrosequencing techniques yielded valuable information on lobster diet.

The bacterial colonies screened from rock lobster faecal samples using the clone libraries did provide several new prey identifications including the temperate Maori octopus (*Octopus maorum*), a species of crab from the southern hemisphere which is known to occur in Tasmania (*Dromia dehaani*), a temperate species of Criniodea (*Florometra serratissima*) and an uncultured Archaea. It is important to note that the sequence matches for *Dromia dehaani* and *Florometra serratissima* were not exact which suggests that the actual prey species ingested by the rock lobster are presently un-sequenced and not in any of the genetics databases. The Archaeon sequence which was detected in this clone library is likely to be an endosymbiont which resides in the gut of the rock lobster as members of this diverse taxonomic group are known to fill this niche (Lange et al., 2005). The sequences from rock lobster may be a confirmation of cannibalistic behaviour which has been previously reported in this species (Mills et al., 2006) or the ingestion of the moulted exoskeleton of a conspecific, but it was not possible to determine with this clone library experiment if the rock lobster DNA is from a predation event or DNA from the predator itself. Understanding the role of cannibalism can be important as it can be a food source or a feedback mechanism for regulating population size.

The pyrosequencing data also added new dietary information. There were several notable parallels to recent stable isotope work on rock lobsters, with the inclusion of novel rock lobster

dietary items such as gastropods (*Prothalotia lehmanni*, *Zethalia zelandica* and *Austrocochlea odontis*), filter feeding invertebrates (Sponges) and red algae (Guest et al., 2009).

It is important to note that the adult phase of some species in these inventories is unlikely to be predated on by rock lobsters, particularly larger fish such as the swell shark, and octopus. However, it is possible that the lobsters predate the egg cases or newly emergent young of the oviparous swell shark. Similarly the identification of *Octopus maorum* in both the clone library and pyrosequencing analysis is noteworthy as this species has never previously been documented in the diet of rock lobsters, and the commonly held belief is that *Octopus spp.* are predators on rock lobsters (Harrington et al., 2006). Octopus are major predators of lobsters in traps and as a result fishers have created a fishery for *Octopus spp.* as a byproduct (Brock and Ward, 2004, Groeneveld et al., 2006). Results from this pilot scale trial suggest that there is a feedback mechanism whereby lobsters also predate on octopus. Whether this is on juveniles, egg masses or naturally killed octopus is uncertain. The soft bodied nature of all *Octopus spp.* would render their tissues unidentifiable in any previous lobster diet study and this is the most likely explanation for their previous absence in the literature.

It is particularly interesting that over all of the samples listed in Tables 5-11 fish species are the most diverse group of prey. Many of these fish species are active during the day and exhibit resting behaviour at night. In contrast, lobsters are generally active during the night with their foraging period overlapping the time when the fish species would be expected to be most vulnerable. However, the fish species identified varied between fished and unfished regions. For example, the wrasses (*Notolabrus tetricus* and *N. fucicola*) are the basis of a commercial fishery and there was a lower frequency of these wrasse species in lobster diets in fished regions indicating a link between lobster diet and the effects of fishing on ecosystem dynamics. This is further demonstrated by the larger percentage of urchins in the diet of lobsters outside the

reserve as studies within the MIMPA have shown that urchins have declined in abundance as lobsters have increased (Barrett et al., 2003).

There has also been considerable speculation about the role of rock lobsters and the foraging behaviour of these predators away from dense reef areas (MacDiarmid et al., 1991, Langlois et al., 2006). Within the samples collected inside MIMPA there were several prey items which are associated with soft sediment (*Echinocardium cordatum*) and seagrass habitats (*Neoodax balteatus*) which would indicate that either the larger lobsters were moving off the reef, as reported in New Zealand (Langlois et al., 2005) or that with the increased population of lobsters in the MIMPA, broader foraging regions are required to obtain sufficient food.

Molluscs were a higher percentage of the diet in the MIMPA than fished regions and these consisted of several species of Trochidae gastropods (*Prothalotia lehmanni*, *Zethalia zelandica* and *Austrocochlea odontis*). This is consistent with previous work in Tasmania and further highlights the interaction between these trophic levels (Guest et al., 2009).

From an ecological perspective, it has been reported that lobsters prey on species from higher trophic levels inside Marine Reserve locations when compared to heavily fished sites (Guest et al., 2009). Although only a small number of lobsters were sampled (n=33), the broad range of species and taxonomic groups recorded in the faecal material would suggest that these lobsters were opportunistic predators and scavengers. If further sampling confirms these results then it is unlikely that lobsters would provide top-down control of specific species through predation, rather they are more likely to minimise the potential for any particular species to dominate a reef region.

In marine ecosystems the differences between predator abundances in fished and unfished locations can be attributed to a wide range of factors including the ecology of the area as well as the behaviour of prey across location (Pande et al., 2008, Babcock et al., 2010b, Micheli et al.,

2004). This data suggests that both seasonal, gender and site differences in diet can be inventoried and compared using a molecular approach. The results show that sea urchins of several species are more common in the diet of lobsters in Marine Protected Areas while octopus species appear to be more prevalent in the diets of lobsters in fished locations. Conversely the detection of bait in the diet of lobsters caught in Marine Protected areas would suggest that greater competition exists for prey items, resulting in preferential consumption of supplemental food resources available in baited lobster traps. It also appears that there is greater prey diversity present in the Marine Protected Area samples than in lobsters foraging in fished locations. This may be due in part to the greater density of rock lobsters in Marine Protected areas which necessitates a more broad foraging strategy in order to consume sufficient resources for daily metabolic requirements. By comparison the lobsters foraging in fished locations appear to be targeting fewer species of prey- perhaps suggesting that the feeding behavior is directed at prey resources in greater abundance at these sites. Several authors have suggested that species diversity increases in Marine Protected Areas (Halpern and Warner, 2002, Lester et al., 2009, Guidetti, 2006) and this theory is certainly supported by the dietary data of rock lobster prey consumption in Tasmanian waters.

There are also noteworthy differences between sexes of lobsters from the same sample location and time. Female lobsters appear to be targeting the bait in the traps at a considerably higher percentage than do the males. This could be explained by the sex ratio of lobsters in the more densely populated Marine Protected areas. As the male lobsters are primarily the target of the commercial and recreational fisheries and the fishing season on male lobsters is longer, many fished locations have considerably higher abundances of female lobsters.

Although based upon a small sample size and a limited temporal snapshot, the lobster dietary samples did show differences between fished and non-fished locations which, while preliminary,

are insightful for understanding the dynamics between these locations and support previous studies (e.g. stable isotope analysis, (Guest et al., 2009) and observations (e.g. species changes in Maria Island Marine Protected Area, (Barrett et al., 2003)). The consistency across the samples also demonstrates the potential of trophic feedback mechanisms by lobsters predating species such as swell sharks and octopus that had previously been considered to be predators of lobsters.

All of the prey identifications add to the overall knowledge of rock lobster behaviour and feeding ecology including off-reef feeding in marine reserves. This confirmation of the value of DNA dietary analysis to provide detailed information from only a small sample demonstrates the potential of the method. It is important to note that each sample only represents the diet of a few individual predators at a very brief snapshot in time from each location. However, the ability to screen large numbers of samples non-destructively opens the way for understanding the role of lobsters in reef systems globally where they are often a key species.

The development of the 454 pyrosequencing techniques has opened up the potential for substantially larger runs of samples and, given the considerably less sample handling by eliminating the need to insert amplified DNA fragments into bacteria, this technique is likely to be the best option in the future. Additionally, the 454 pyrosequencing data flow delivers 'deep' sequencing and the exhaustive nature of the technology can yield considerably more information for each individual sample (Edwards et al., 2006, Deagle et al., 2009). Importantly for future research in this area, the costs associated with the 454 pyrosequencing technique are decreasing as more laboratories become equipped with the necessary apparatus and technical expertise to analyse the data.

There are, however, presently some limitations with any library creation due to the reliance on genetics databases. All species in these databases need to be accurately identified and sequenced prior to being entered into the public arena (Hebert et al., 2003b). Thus the databases are normally better for the well-known species, especially the conspicuous vertebrates, but often

poor for the lesser known, rare and unidentified organisms. The databases are increasing all the time and as a result it is often possible to determine an appropriate species or family if a close relative has already been identified and sequenced, even if no identifications have been submitted for members of that “type” in the specific region being analysed. This is a common occurrence in the more sparsely studied southern temperate marine environment.

This analysis has demonstrated the potential for DNA to provide detailed information on the diet of lobsters. Importantly, the use of non-lethal faecal material (Redd et al., 2008) enables samples to be obtained from marine protected areas and from a fishery where the commercial product is sold live and of high value. These results demonstrate the potential of dietary analysis to provide detailed information that has not previously been possible on lobster diet. The large number of samples that can be processed through the 454 pyrosequencing method can be used to examine dietary preferences from multiple regions across the fishery and to compare within and between protected and non-protected areas-the latter being important for the study of the impacts of climate change and the effects of fishing on marine resources.

6.6 Acknowledgements

This research was a component of the grant: FRDC 2004/013 “Towards integrated multi-species management of Australia’s SE reef fisheries: A Tasmanian example.” which was supported by funding from the Fisheries Research and Development Corporation on behalf of the Australian Government. We would additionally like to thank all the individuals from the IMAS, Taroona Marine Research Laboratories and R/V Challenger who assisted with rock lobster potting and collection at the Marine Island Marine Protected Areas, as well as around the fished locations at Bicheno and Recherche Bay. Particular thanks go to Dr Hugh Pederson who facilitated the winter trips to the Marine Protected Area at Maria Island as well as the dedicated students from the

UTAS Marine Ecology (KZA 356) Unit who helped with the capture of the rock lobsters during their course. All lobster experiments were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 7th Edition 2004.

Chapter 7

Applications of molecular prey detection techniques to understand trophic linkages in marine systems

Parts of this chapter have been published in:

Barnett, A., Redd, K., Frusher, S., Stevens, J. & Semmens, J. (2010). Non-lethal method to obtain stomach samples from a large marine predator and the use of DNA analysis to improve dietary information. *Journal of Experimental Marine Biology and Ecology*, 393, 1-2, 188-192.

Roura, Á., Guerra, Á., Redd, K., González, Á. (2012) Molecular prey identification in wild *Octopus vulgaris* paralarvae. *Marine Biology*, 159, 6, 1335-1345.

I was responsible for organising, analysing and interpreting the DNA component of each of these publications

“...And a bright ideal, tomorrow
Don't go too far
Stay who you are

Everybody knows
You only live a day
But it's brilliant anyway....”

Independence Day

Elliott Smith

XO

1998

Chapter 7: Applications of molecular prey detection techniques to understand trophic linkages in marine systems.

7.1 Introduction

Resolving diets of animals immersed in complex food webs in marine ecosystems is extremely challenging (Mayfield et al., 2000c, Nyssen et al., 2005) particularly when the animals are small, cryptic, nocturnal or residing in complex benthic or pelagic habitats. Direct observations of predation events are rare and may even fail to capture the normal behaviour of most of these animals over time (Pederson and Johnson, 2006, Sheppard and Harwood, 2005). There are several well documented methods to identify prey consumed by animals, most of which require field collection and postmortem analysis of the digestive tract and morphological prey analysis of the ingested and macerated prey species (Rosel and Kocher, 2002, Williams, 1981, Hakala and Johnson, 2004). The development of molecular methods has provided increased resolution (Symondson, 2002, King et al., 2008a) in both the variety of prey items and their contribution to diet. This chapter describes three very different applications of dietary DNA analyses to demonstrate the broad applicability of these techniques to provide insights into and to enhance our knowledge of trophic linkages in marine systems. This is demonstrated through analysis of a key reef based commercial invertebrate species (southern rock lobster – *Jasus edwardsii*), a small pelagic paralarvae (*Octopus vulgaris*) and large vertebrate predator from a protected region (seven gilled shark - *Notorynchus cepedianus*).

These species present different rationales for the exploration of their diet as well as the techniques necessary to determine their prey consumption patterns and represent both the

versatility and diversity of potential options using molecular methods. Firstly the southern rock lobster (*Jasus edwardsii*) was selected as an important temperate reef predator about which much speculation had arisen as to the trophic role of this animal in temperate reef ecosystems (Andrew and MacDiarmid, 1991, Kelly et al., 2000). Previous work in California, New Zealand and South Africa suggested that many Palinurid crustaceans were key predators exerting top down regulation of kelp dominated nearshore systems (Barkai et al., 1996, Guest et al., 2009, van Zyl et al., 1998, Tegner and Levin, 1983a, Foster and Schiel, 2010). Due to the valuable nature of these crustaceans, any dietary samples from the lobsters needed to be collected from live animals in a non-lethal manner. This necessitated the development of a method to obtain non-lethal samples which was achieved by obtaining faecal material (Redd et al., 2008). Importantly, the faecal material is assumed to contain a complete mixture of dietary intake, an issue that has hindered previous studies where the digestive processes of lobsters has limited the ability to identify many species (especially soft bodied species) through post-mortum examination of gut contents (Mayfield et al., 2000c). For lobsters, knowledge was required on specific predator prey interactions as well as an increased understanding of the breadth of lobster diet. Secondly, the seven gilled shark (*Notorynchus cepedianus*) was chosen as predator/scavenger about which very little was known. The capturing, tagging and tracking of these sharks involved the non-lethal collection of stomach content materials through stomach flushing and evacuation (Barnett et al., 2010a). These sharks were captured in a nursery zone where killing of sharks was illegal, therefore the ability to collect dietary information from species that are being used for multiple studies (e.g. tagging and tracking studies) and conservation necessitated the return to the sea alive of every individual captured. Furthermore, many of these species are in low relative numbers and maximising the information from each individual is required. After initial separation of the flushed material into pieces that could be visually identified, there remained sections of prey that were either indistinguishable to species level or completely indistinguishable. For this shark species, where only a few samples were available, maximising the information from each sample

was important. Thirdly, understanding the prey of very small pelagic animals requires microscopic analysis of the gut contents and knowledge of phyto- and zooplankton species – several of which can be rapidly digested. Octopus (*Octopus vulgaris*) paralarvae, which had been previously collected from Spanish waters were investigated to determine the suitability for understanding planktonic trophic linkages.

7.2 Overall methodology

In order to identify either general or specific dietary components of any marine animal to a high taxonomic level, the initial development of any molecular method must be considered carefully (Symondson, 2002, King et al., 2008a). Using species-specific or taxonomic group specific PCR primers to score the presence of known prey by PCR amplification can be validated with captive feeding experiments (Redd et al., 2008, Roura et al., 2010). Next, a molecular method must be tested for the detection and quantification of the natural prey of animals collected in the field (Barnett et al., 2009, Deagle et al., 2007, Jarman et al., 2004, Casper et al., 2007b). Because there are so many potential prey species in the marine environment, trying to resolve the entire diet using species-specific primers is seldom feasible as it is potentially prohibitively time-consuming and virtually impossible to create single-species primers for every possible invertebrate in any given marine bioregion (Blankenship and Yayanos, 2005). It is, however, possible to answer a direct ecological question about the role of the predator by creating a very specific molecular assay – eg. examining the relationship between the predator and one or more key prey species (King et al., 2008a). This approach was applied to understand the interactions between rock lobsters and sea urchins (see Chapter 5). Additional challenges occur when working with a small sized predator such as *Octopus vulgaris* paralarvae which have a high metabolic rate and very efficient digestion. For this system, it was necessary to develop techniques which would be both stringent and sensitive for low levels of prey DNA. The ratio of prey: predator DNA was also likely to cause challenges to the kinetics of the PCR reaction and increase the potential to amplify

predator DNA which would mask the presence of any available prey DNA. A nested PCR approach was chosen for this system to initially amplify all non-predator DNA and then to undertake a second PCR for groups of potential prey species. Finally, a more conventional 'forensic' approach was applied to the seven gilled shark (*Notorynchus cepedianus*) system where relatively large pieces of prey could be collected and sorted into 'rough' taxonomic groupings. For this work, the sorted prey items were used as an inventory of the sharks' dietary consumption and molecular tools were used to provide exact species level identifications for all 'known' and 'unknown' prey material which had been recovered by non-lethal stomach pumping methods (Barnett et al., 2009).

7.3 Genetic methodology rationale and overview

All eukaryotic cells contain mitochondria as mitochondria play an essential role in cellular metabolism (Wilson, 1999). These organelles contain multiple copies of circular DNA, the mitochondrial genome, often referred to as mitochondrial DNA (mtDNA) which typically encodes rRNA, tRNA, and numerous important cellular polypeptides (Freeland et al., 2011). These polypeptides are responsible for the production of protein complexes in the inner mitochondrial membrane which facilitate the production of ATP (Wilson, 1999). Based upon genomic similarities, gene regions from within the mitochondrial genome; Cytochrome C Oxidase I (COI), Cytochrome b (Cob), 12S rRNA and 16S rRNA have risen to prominence for phylogenetic studies (Liu et al., 2011, Allcock et al., 2011), barcoding applications (Vences et al., 2005, Ward et al., 2005) and prey detection work (Symondson, 2002, King et al., 2008a, Ward et al., 2005). The mitochondrial 16S ribosomal RNA gene (16S rRNA) has been used successfully for designing group-specific primers which amplify this small region of the mitochondrial genome (Kasper et al., 2004). This region is highly informative and well suited to use for dietary analysis (Deagle et al., 2005b,

Blankenship and Yayanos, 2005). Another mitochondrial gene region, Cytochrome Oxidase (COI), has been commonly chosen for barcoding, and has therefore attracted many sequences to the global genetics databases (Hebert et al., 2003b). This region, however, is not necessarily ideal for designing species-specific primers as the variations between species and even groups of species is often only a few nucleotides (Nielsen and Matz, 2005, Vences et al., 2005), but nonetheless numerous authors have used it for prey detection purposes (Dalén et al., 2004, Farrell et al., 2000, Foltan et al., 2005). These subtle variations are more ideally suited to species identifications when discrete amounts of prey tissue can be collected and we have used COI for this purpose with shark gut contents (Barnett et al., 2009), but not for the development of specific PCR primers. For these reasons, we primarily targeted small, multi-copy DNA fragments with universal primers from the 16S rRNA gene for prey inventories (Chapter 6), and in conjunction with group-specific primers designed to anneal short target templates of potential prey items, which would not amplify predator DNA for our nested PCR approaches (Roura et al., 2012). The nested PCR approach does, however, require *a priori* knowledge of the fauna that coexist with the predator. Fortunately, the GenBank and Barcode of Life databases have been well populated with relevant fish and invertebrate sequences, and there is a solid framework on which to base primer design (Ward et al., 2005, Holmes et al., 2008, Hebert and Gregory, 2005) with submitted sequences and thorough identifications increasing all the time.

7.4 Molecular methods

Sample collection and DNA extraction

The first step in developing any molecular based assay is to collect dietary material from the predator (Symondson, 2002). This can be done lethally by directly removing the digestive tract and contents by dissection (Passmore et al., 2006) or in the case of small predators such as larvae, the entire predator can be analysed (Chow et al., 2006, Roura et al., 2010). If a non-lethal approach is pursued (such as when the material is obtained from a high value live market commercial species (e.g. rock lobster) or when working in protected regions such as Marine Protected Areas or shark nursery grounds (e.g. seven gilled shark)) then it is necessary to use or develop methods that collect faecal material or stomach contents without killing the predator (Deagle et al., 2007, Dalén et al., 2004). Lobster samples were obtained non-destructively by removing faecal material from the anal pore (Redd et al., 2008) and shark gut samples were collected by gastric flushing prior to the shark being released (Barnett et al., 2009).

Gut content or faecal DNA must then be isolated from cellular material and other digestive compounds. The DNA purified from stomach contents or faeces is likely to be a mixture of bacterial DNA (from microbial gut symbionts), predator DNA and the target prey DNA (Symondson, 2002). The DNA from prey is highly degraded due to digestive processes (Deagle et al., 2006) but there are presently a wide range of commercial 'kits' which are designed to deliver consistent results and to yield quality DNA (Foran, 2008). Most of these 'kits' contain proprietary reagents which have been modified from well-known laboratory protocols (MoBioLaboratories, 2003, QIAGEN, 2004). It is, however, necessary to determine the most suitable procedure to achieve the highest DNA yields from each type of dietary sample. It is also important to ascertain which 'kit' will remove any potential compounds which would inhibit the PCR reactions and other

‘downstream’ applications (Juen and Traugott, 2006). For the seven gilled shark analysis, a more cost effective DNA extraction approach was chosen with the QIAGEN DNeasy[®] Kit (QIAGEN, 2004) and due to the small size of the *Octopus vulgaris* paralarvae digestive tracts the QIAamp DNA Micro Kit (QIAGEN, 2008) was chosen for this system. For lobster faecal samples, the biochemical nature of the faecal pellets was most like that of soils and therefore The Ultra Clean™ Fecal/Soil DNA Kit (Mo Bio Laboratories, Inc.) was chosen.

With this Ultra Clean™ Fecal/Soil DNA Kit, the faecal samples are loaded into a bead tube with sterilized beads designed to mechanically disrupt cellular membranes. This is the first part of the protocol which takes place in a buffer designed to disperse the faecal particles and begin the process of cell lysis with sodium dodecyl sulphate (SDS). The SDS detergent breaks down fatty acids and lipids associated with the cell membrane of most eukaryotic organisms and the addition of a proprietary reagent (referred to as IRS) is intended to precipitate other PCR inhibitors which commonly occur in dietary samples. This precipitation step was extremely beneficial for the downstream use of the DNA in PCR-based applications explored in these dietary applications. After extensive mechanical and chemical lysis, the prey and predator cells have been broken open and then a centrifuge step is used to precipitate particulates of cell debris, shell fragments, sediment, beads, and humic acids into a pellet. At this stage, the DNA is in the liquid supernatant which is removed from the precipitated pellet. The next steps involve further precipitation of remaining dissolved proteins with the addition of glacial acetic acid and a step to introduce chaotropic salts to denature proteins. Under these conditions, the DNA selectively binds to the silica resin in a manufactured spin column, allowing the rest of the cellular material to be separated by centrifuge. When the DNA is selectively bound to the silica membrane in the spin filter device, most contaminants pass through the filter membrane, leaving only the desired DNA behind. At this point in the protocol, several ethanol based wash solutions are used to further

clean the DNA that is bound to the silica filter membrane in the spin filter column. These wash solutions remove residues of salt, humic acid, and other contaminants while allowing the DNA to stay bound to the silica membrane. The final DNA isolation takes place by elution -removing the desired DNA from the silica membrane. Once the DNA is released, it flows through the membrane, and into the collection tube. The DNA is released because it can only bind to the silica spin filter membrane in the presence of salt and the final elution buffer, 10 mM Tris pH 8 does not contain salt.

For smaller samples including *Octopus vulgaris* paralarvae, the DNA was extracted with a QIAamp DNA Micro Kit (QIAGEN), using an RNA carrier in the initial lysis buffer. All steps followed manufacturer's detailed protocols and instructions which employ the same basic chemistry as the The Ultra Clean™ Fecal/Soil DNA Kit, with the exception of the lysis step. In the QIAamp DNA Micro Kit, the lysis was not done mechanically with beads and SDS, but instead with a protein degrading enzyme, proteinase K, and an incubation step at 56°C which was done overnight. Also the silica membrane in the QIAamp DNA Micro Kit (QIAGEN) in conjunction with the RNA carrier was considerably more efficient at isolating and binding the smaller amounts of DNA present in paralarvae. This extraction protocol proved to be extremely beneficial for this application.

For larger samples such as the shark gut contents, discrete pieces of prey tissue could be easily physically isolated from one another and sorted visually (Barnett et al., 2009). With these much more substantial samples, a small subsample was taken from inside the larger piece of prey tissue, which avoided the potential contamination from predator DNA. This situation necessitated a less robust DNA extraction protocol and the DNeasy® Tissue Kit (QIAGEN) was used for all of these samples.

Specific PCR primer design

Universal primers amplifying mitochondrial DNA (mtDNA) have been broadly used in phylogenetic studies (Inoue et al., 2007, Matthee et al., 2007) and in prey detection applications (Symondson, 2002, Kasper et al., 2004). The 3' end of the mitochondrial ribosomal 16S gene is particularly useful for these purposes as many sequences have been submitted to the international GenBank database (NCBI, 2006) and Barcode of Life database (Ratnasingham and Hebert, 2007). This mitochondrial region varies in size depending on the taxa because of insertions and deletions-enabling accurate taxonomic resolution and the identification of unknown sequences to species or genus. Numerous authors on molecular prey detection have successfully used this region to design group-specific and species-specific PCR primers (Deagle et al., 2005a, Deagle et al., 2005b, Jarman et al., 2004, Jarman et al., 2006). In order to avoid the amplification of the predator DNA, group-specific primers and species-specific primers can be designed using 16S rRNA sequences from GenBank by choosing a wide range of potential prey items which are then aligned to find regions of DNA within the target group of potential species where the primers would bind strongly but where predator DNA would not be amplified (Jarman et al., 2004). The software AMPLICON (Jarman, 2004) was used to identify the aligned regions which were likely to be useful as group specific primers in both the rock lobster and *Octopus vulgaris* paralarvae components of this thesis. Primer specificity must always be tested by PCR using a gradient of annealing temperatures on known template DNA from across the range of potential prey species as well as on predator DNA template as documented by numerous authors working in this field (Admassu et al., 2006, Chen et al., 2002, Jarman et al., 2006).

Table 1. PCR primers used in the current study showing the name, sequence of both forward and reverse primer, DNA target region, target group, expected amplicon size and the first published appearance of each primer.

Primer	Sequence (5'–3')	DNA region	Primer Specificity	Target species/group	Amplicon size	Publication
LR-N-13396 (16s nr)	CGC CTG TTT ATC AAA AAC AT	16s	Universal	Invertebrates	500-650	Folmer, et al 1994
LR-J-12887 (16s nr)	CCG GTC TGA ACT CAG ATC ACG T	16s	Universal	Invertebrates	500-650	Folmer, et al 1994
FishF1	TCA ACC AAC CAC AAA GAC ATT GGC AC	COI	Class	Telostei and Chondrichthyes	655	Ward, et al 2005
FishR1	TAG ACT TCT GGG TGG CCA AAG AAT CA	COI	Class	Telostei and Chondrichthyes	655	Ward, et al 2005
FishF2	TCG ACT AAT CAT AAA GAT ATC GGC AC	COI	Class	Telostei and Chondrichthyes	655	Ward, et al 2005
FishR2	ACT TCA GGG TGA CCG AAG AAT CAG AA	COI	Class	Telostei and Chondrichthyes	655	Ward, et al 2005
HalCO2GENA	CAA TYT GAA CYA TTC TMC CAG C	COII	Species	<i>Haliotis rubra</i>	193	Conod, et al 2002
HalCO2GENB	CCT TAA ART CTG AGT ATT CGT AGC C	COII	Species	<i>Haliotis rubra</i>	193	Conod, et al 2002
EchinNSSf1	GCG TGC TTT TAT TAG GA	18s	Phylum	Echinodermata	160	Jarman, et al 2006
EchinNSSr1	CGA CCA TGR TAR GCG CAT AAC G	18s	Phylum	Echinodermata	160	Jarman, et al 2006
GastNLSf1	GCG GYA ACG CAA ACG AAG T	18s	Phylum	Cephalopoda	188-198	Jarman, et al 2006
GastNLSr1	CGA AAW TMA CAC CGT CTC TCC G	18s	Phylum	Cephalopoda	188-198	Jarman, et al 2006
16Sfou	GAC GAT AAG ACC CTA TAA	16s	Superorder	Eucarida	194-204	Roura, et al 2011
16Srou	CGC TGT TAT CCC TAA AGT AA	16s	Superorder	Eucarida	194-204	Roura, et al 2011
Hali16sF	TCA AAG GAA GTT ACC G	16s	Species	<i>Helicidaris erythrogramma</i>	202	Raddi
Hali16sR	CCC TTA AAA GCT TCT GCA CCC T	16s	Species	<i>Helicidaris erythrogramma</i>	202	Raddi
Alba2F	GCT TTT GCT GGA AAA ACT CG	16s	Species	<i>Haliotis rubra</i>	235	Raddi
Alba2R	CGT TCC CCA GTT TTA GGT GA	16s	Species	<i>Haliotis rubra</i>	235	Raddi
Centro 16sF	GGA ACA GCA AAC ATG GAG AGT CCT GC	16s	Species	<i>Centrostephanus rodgersii</i>	161	Raddi
Centro 16sR	CCG TCT TGC CAT TCA TGC CAG TCT CTA	16s	Species	<i>Centrostephanus rodgersii</i>	161	Raddi
CENT 2 F	GGG CAA CCG CGG AGA AGG AAT ATC C	16s	Species	<i>Centrostephanus rodgersii</i>	268	Raddi
CENT 2 R	CGC AAA CCT TTT TGT CAA	16s	Species	<i>Centrostephanus rodgersii</i>	268	Raddi
Hali16sF	GGG GTG ACT GGG GAA CAA T*AGT	16s	Genus	<i>Haliotis</i> sp.	149	Raddi
Hali16sR	CTA CAC CCT CAG GAC ACC TTA ATCC	16s	Genus	<i>Haliotis</i> sp.	149	Raddi
Plagi16sF	GAC *GT GCA AAG GTA GCA TA	16s	Genus	<i>Plagusia</i> sp.	400	Raddi
Plagi16sR	TAA TTC AAC ATC GAG GTC GCA	16s	Genus	<i>Plagusia</i> sp.	400	Raddi
Pallidus16sF	CTC GGT GAG ATA TAA	16s	Species	<i>Octopus pallidus</i>	147	Raddi
Pallidus16sR	GTC CCT TTA A	16s	Species	<i>Octopus pallidus</i>	147	Raddi
Maorum16sF	GCT CGG TGA GAA TAA AAT	16s	Species	<i>Octopus maorum</i>	173	Raddi
Maorum16sR	GTC CCT TTA AA	16s	Species	<i>Octopus maorum</i>	173	Raddi
Jan16sF	TCA AAT ATC CT* GGG GGA CGA TAA GAC CCT ATA	16s	Species	<i>Janus edwardii</i>	259	Raddi
Jan16sR	CCT TGC CTT CGA TAA GGA CTC TC	16s	Species	<i>Janus edwardii</i>	259	Raddi

Genetic databases

Occasionally while searching for rare predator (such as southern hemisphere marine invertebrate prey species) sequences in GenBank, relatively few matches would be made from particular taxonomic groups and therefore it was necessary to use sequences from related organisms from other regions. Quite often species from Asia and the Pacific were available and these would be used preferentially to Atlantic species whenever possible if working on Australian predator/prey systems such as the southern rock lobster and seven gilled shark (*Notorynchus cepedianus*). To ensure the correct sequences were used to design species-specific PCR primers, mtDNA16S

sequences were obtained from local sea urchins, (*Centrostephanus rodgersii* and *Heliocidaris erythrogramma*) for the detection of these species in rock lobster faecal material and from Atlantic zooplankton species sourced from plankton tows in the Spanish region for the detection of these species in *Octopus vulgaris* paralarvae. These species were collected, visually identified, and DNA was extracted from fresh tissue with the DNeasy® Tissue Kit (QIAGEN) following the manufacturer's protocol for animal tissues. PCR products were generated with the universal primers 16sar (5'-CGC CTG TTT ATC AAA AAC AT-3') and 16sbr (5'-CCG GTC TGA ACT CAG ATC ACG T-3') with the resulting amplicons ranging from 500bp-650bp. This approach may be necessary for developing new and novel prey detection assays where insufficient sequence data is available.

To identify the diversity of species in seven gilled shark diet (*Notorynchus cepedianus*), the combination of GenBank and BOL genetic databases resulted in exact species level identifications in most cases, avoiding the challenges associated with poor species matches (Barnett et al., 2009). This is a result of considerable efforts to resolve the taxonomy and to subsequently barcode the fishes of Australia (Ward et al., 2005). This increased taxonomic resolution resulted in improved information on the frequency of occurrence of several species in the diet and therefore the importance of species-specific trophic linkages. Morphological analysis had identified *Mustelus antarcticus* as a relatively important prey species of *Notorynchus cepedianus*, however DNA analysis showed that *Notorynchus cepedianus* had actually consumed twice the number of *Mustelus antarcticus*, emphasising the importance of predator-prey link between these species (Barnett et al., 2010b). By undertaking molecular analysis of unidentifiable prey and using the combination of GenBank and BOL genetic databases, the number of species with precise identification was doubled. This resulted in a better understanding of both the diversity of prey items consumed and the importance of specific prey species to *Notorynchus cepedianus* diet.

Polymerase chain reaction (PCR) platform

Most PCR reactions are performed with a single set of primers to establish a binary result for the presence or absence of the DNA in a given sample or to sequence the unknown amplicons for species level identification (Altshuler, 2006). This basic approach generally necessitates an electrophoresis step to confirm the amplification (McPherson, 1991). This additional step can be avoided by using a quantitative Real Time PCR (qPCR)-based platform to track the amplification reaction in real time (Bustin, 2004, Weber and Lundgren, 2009b, Perkel, 2006). For dietary assays, additional quantitative information is also generated by using qPCR and a SyBr green dye in conjunction with species specific primers, without the need for electrophoresis (Deagle and Tollit, 2006).

A more complex approach to PCR may be required in cases where predator DNA greatly overwhelms potential prey DNA in the amplification reaction (Roura et al., 2012). In order to detect prey DNA inside the digestive tract of the paralarvae a nested PCR was performed by using group-specific primers for the first amplification and then using the resulting PCR product (diluted five times) as template for a subsequent PCR with species-specific primers (Roura et al., 2012). This approach was necessary to overcome the abundance of predator DNA and highlights the challenges associated with homogenizing the entire predator and extracting DNA from the whole organism. Although the nested PCR approach was used successfully for resolving the diet of *Octopus vulgaris* paralarvae there are other techniques that are based on amplifying the stomach content with universal primers accompanied by a secondary analysis to distinguish between the different sequences obtained and also novel methods of 'blocking' the amplification of predator DNA while amplifying only the prey DNA templates (Blankenship and Yayanos, 2005, Chow et al., 2011, Dunshea, 2009, Vestheim and Jarman, 2008). These techniques have considerable

potential to improve the amplification efficiency of any molecular prey detection experiment and as such are worthy of considering when predator DNA has the potential to “swamp” the analysis.

Quantification of prey: qPCR

The ultimate goal of many predation studies is to develop a full prey inventory and to quantify the abundance of each prey item in the diet of the predator. To fully understand the diet of wild animals using molecular tools, a portion of the goal of prey quantification can be achieved by starting with a model system and conducting rigorous feeding experiments to validate prey intake under controlled conditions (Deagle et al., 2006, Deagle et al., 2005b). There are, however, significant challenges when using these experiments to interpret samples collected from wild predators. If a degraded dietary sample contains low concentrations of prey DNA, the exact source of this material cannot easily be resolved by molecular analysis. For example, a predator may have consumed a small amount of prey material quite recently, or may have ingested a larger amount of prey material a longer time ago. Both of these scenarios have the potential to show low prey DNA concentrations despite the later prey item potentially being a major contributor to the diet. These ambiguities aside, the overall detection rates as ‘presence or absence’ (binary data) can be quite informative. Presence and absence data can be obtained much more quickly and efficiently by using the qPCR platform because the electrophoresis step after PCR is not necessary.

Identification of prey from mixed templates: DNA cloning and sequencing

The isolation of unique DNA molecules from mixed, complex environmental samples has always been problematic for molecular biologists (Satokari et al., 2003, Edwards et al., 2006, Rappe et al., 1997, Bowman and McCuaig, 2003). Initial steps in the process require the amplification of DNA by PCR and then either; cloning the amplicons into plasmids for growth in bacteria on agar plates

(Dorigo et al., 2002) or using next generation sequencing technology (Deagle et al., 2010, Deagle et al., 2009, Diggle and Clarke, 2004, Edwards et al., 2006). Both of these approaches are presently time consuming and relatively expensive to perform, rendering them relatively impractical for large numbers of field samples and broad scale dietary analysis. These techniques are, however, important tools when commencing a new prey inventory study and therefore may be required.

For the work in this thesis, several clone libraries were made using PCR products from rock lobster faecal samples (Chapter 6) and the digestive tract of the *Octopus vulgaris* paralarvae (Roura et al., 2012). The results from the *Octopus vulgaris* paralarvae experiments revealed several new prey identifications and broadened the understanding of trophic relationships in the mesozooplankton (Roura et al., 2012). The colonies screened from rock lobster faecal samples yielded several new prey identifications including *Octopus maorum* and a previously un-sequenced Criniodea (Chapter 6). Both of these prey identifications add to the overall knowledge of rock lobster feeding ecology but only represent the diet of individual animals at a very particular snapshot in time. For large numbers of rock lobster faecal samples, this clone library approach was not feasible for developing a comprehensive dietary inventory and therefore pyrosequencing was employed using the next generation/pyrosequencing/454 platform (Chapter 6).

As new high-throughput sequencing technologies such as pyrosequencing, are being increasingly used for dietary analysis, the enormous amount of data obtained (Deagle et al., 2010, Deagle et al., 2009) presents a major challenge to manage. There are, however, considerable benefits of pyrosequencing when compared to the clone library approach. Many more samples can be efficiently screened and broad inventories created without having to grow bacterial colonies. It is

important to note that this pyrosequencing technology is still limited by the specificity of the primers employed as in all amplification based methods.

Sample bias towards DNA of predators: a pitfall of cloning and sequencing

There is often considerable predator DNA in any mixed dietary sample (faeces, vomit, stomach content) which has the potential to 'swamp' downstream molecular analysis (Deagle et al., 2005a, Casper et al., 2007b, Chow et al., 2011, Farrell et al., 2000, Weber and Lundgren, 2009b). This is not necessarily a problem with the newer pyrosequencing platform, as this technology can deliver thousands of sequences per sample giving sufficient data on dietary composition even with the large numbers of predator sequences (Raye et al., 2011). Several authors have reported on the use of blocking primers (Vestheim and Jarman, 2008), nested PCR (Roura et al., 2012) and suicide polymerase techniques (Green and Minz, 2005) to eliminate the more dominant/predator sequences in dietary samples, but for this work on the rock lobster, these approaches were not deemed appropriate. The primary reasons for this were the lack of knowledge about the other organisms in the rock lobster diet and the goal of developing a complete inventory of all items consumed by this predator across locations and times. Many benthic invertebrates from temperate Australia which have the potential to be lobster prey items were either not present in the databases for use in developing blocking primers or nested PCR. Additionally there is a high likelihood that other decapod crustaceans were a dietary component of the rock lobster diet as shown in the preliminary clone library (Chapter 6).

In contrast, for work on the *Octopus vulgaris* paralarvae (Roura et al., 2012) the nested PCR approach was chosen as the likelihood of other cephalopods in the diet was very low and the group-specific PCR primers could easily be designed for all other zooplankton in the region. All

octopus digestive tracts yielded amplifiable DNA with the primer sets used suggesting that the digestion process had not entirely degraded the prey material but this result did not eliminate the possibility that predator DNA was being amplified. When the complete library of 118 clones were sequenced, 115 readable sequences were obtained but only three clones (2.5%) could be attributed to prey as all the other amplicons corresponded to the band of the first PCR (approximately 370 bp), which was not specific for the known prey species. When using the nested PCR approach and the full library of 112 clones were sequenced, all clones corresponded to the nested PCR band and were 100% specific for prey species with no clones from the predator. These results clearly demonstrate the successful use of nested PCR to eliminate the large amounts of predator DNA often associated with dietary samples. This is consistent with the reports of other authors (Vestheim and Jarman, 2008, Passmore et al., 2006) and validates this technique for future work where the ratio of predator to prey DNA dominates.

7.5 Pitfalls associated with molecular techniques

Primer design *in silico* based upon sequence availability

Developing both species-specific and group-specific PCR primers can be problematic in environments where complete sequence coverage is not available. In such situations, the design of group-specific primers without complete knowledge of the prey species might eliminate not only predator but also prey DNA from the full range of potential prey species being detected.

A method to overcome this challenge involves the use of restriction analysis with endonucleases targeting predator DNA, leaving prey DNA intact for amplification (Blankenship and Yayanos, 2005, Suzuki et al., 2006, Dunshea, 2009). This approach requires a unique restriction enzyme

cutting site in the predator that is not present in any prey, necessitating *a priori* knowledge of the prey in order to select the endonucleases. This can be accomplished using software designed specifically for the selection of restriction enzyme cutting sites (Jarman, 2006). Another viable option is to selectively block predator DNA from amplification, as in the example of using a predator blocking primer to avoid amplification of the Antarctic krill, *Euphausia superba*, which consisted of an annealing inhibiting primer overlapping with the 3' end of the reverse universal primers for 28S rDNA, but extended into krill-specific sequence and modified with a C3 spacer at the 3' end (Vestheim and Jarman, 2008). More recently authors have applied a lobster-specific peptide nucleic acid (20 nucleotides) designed to anneal at the junction of the 18S rDNA and the internal transcribed spacer 1 (ITS1) to selectively inhibit amplification of the Japanese spiny lobster *Panulirus japonicus* and eel *Anguilla japonica* (Chow et al., 2011). Although blocking methods are promising they have the potential to exclude the amplification of other prey species as well which greatly reduces the utility of the assay for broad species identification. For these reasons, none of the above methods were selected for either the *Octopus vulgaris* paralarvae (Roura et al., 2012), rock lobster (Redd et al., 2008) or seven gilled shark work (Barnett et al., 2009) as described in this thesis.

Variability in PCR primer performance

Group-specific PCR primers were designed to amplify the most common potential prey species from a wide range of marine taxa (Jarman et al., 2006). The 3' end of the mitochondrial ribosomal 16s gene region was selected for designing group-specific primers, because this variable region is widely used for both molecular identification and dietary analysis. Before designing group-specific primers, the universal primers 16Sar and 16Sbr (Simon et al., 1994) were assessed for broad taxonomic screening as they had been successfully applied to identify prey in the guts of

cephalopods (Braley et al., 2010), fur seals and penguins scats (Casper et al., 2007b, Deagle et al., 2005b) and wasps (Kasper et al., 2004). Nonetheless, preliminary tests conducted with DNA extracted from different crustaceans and *Octopus vulgaris*, revealed the strong affinity of these primers for *Octopus vulgaris* DNA, whereas no amplification was obtained from any of the planktonic crustaceans tested. Although “universal” primers supposedly amplify DNA in a wide range of species (Simon et al., 1994), often when PCR is applied to a mixed source of template DNA the reaction can fail (Jarman et al., 2004). This reaction efficiency may be attributed to the dominance of some sequences in the template mix which disproportionally amplify and therefore mask any rare sequences present in lower concentrations. The PCR reaction may also amplify the DNA templates which are of a higher quality, such as intact predator material as opposed to degraded prey remains (Blankenship and Yayanos, 2005). The “universal” PCR reactions carried out here on different planktonic crustacean species suggest that the problem was with the universal 16Sbr primer, because expected amplicons were obtained when the reactions were carried out with the primers 16Sar-16Scrur and 16Scruf-16Scrur, but no amplifications were obtained when 16Sbr was present in both combinations 16Sar-16Sbr and 16Scruf-16Sbr.

Secondary predation and ‘background’ material

Secondary predation occurs when a predator consumes a second predator, shortly after the latter has consumed the target prey and several authors have detected this using molecular techniques (Sheppard et al., 2005). This type of intraguild predation could potentially restrict the interpretation of any data obtained. While this error was negligible in antibody-based systems (Harwood et al. 2001), in a PCR-based study secondary predation was found to be a significant potential source of error four hours after eating the primary predator (Sheppard et al., 2005). However, the importance of secondary predation as a source of error will depend almost entirely upon the digestion rates of the predators involved. In the case of crustaceans, little is known

about the digestion of prey-associated DNA during gut transit, but several works suggest that digestion may be partial and a significant fraction of prey fragments might survive gut transit in copepods (Montresor et al., 2003, Gagnon et al., 2011).

Taking this into account with the *Octopus vulgaris* system (Roura et al., 2012), secondary predation might be a plausible explanation if the paralarvae ingested the copepod shortly after the prey was consumed by the copepod. Nevertheless, there is little direct evidence to substantiate a potential secondary predation event as copepod DNA was not detected inside the *Octopus vulgaris* paralarvae. But local knowledge would suggest the detection of secondary predation may have taken place because copepods are one of the most abundant zooplankton taxa in the Ría of Vigo (comprising 35-56% of the total abundance from July to October). Thus, encounter rate mediated by stochastic movements would suggest that paralarvae and copepods would have frequent encounters and the likelihood of predation events is very high.

The rock lobster system, on the other hand has considerable potential for the detection of both secondary predation as well as the 'environmental' consumption of prey DNA from sediment sources. This is due to the recently reported foraging behaviour of rock lobsters where ingestion of benthic detritus and sediment material is a relatively common occurrence (Langlois et al., 2006). Thus, there is a higher likelihood that this sort of secondary prey detection and/or detection of waste products in the sediment (see Chapter 5) could occur with *Jasus edwardsii*.

Technical capacity and costs associated with molecular biology

The present capacity of international research institutes will enable most (if not all) of the techniques described in this thesis to be carried out 'in house'. There is, however, a level of

technical proficiency required to perform the molecular assays and appropriately skilled personnel are necessary to successfully undertake any new prey detection project. Although the reproducible nature of molecular biology has been greatly increased with the advent of 'kit' based DNA/RNA extraction as well as PCR and qPCR reagents, these proprietary chemicals are notoriously costly, putting them out of range to projects lacking sufficient funds.

7.6 Benefits associated with molecular techniques

Primers and software are widely available

Taxon-specific DNA markers can be designed by identifying short DNA regions unique to a given prey species as described throughout this thesis. Presently, however, there are so many group-specific, species-specific and 'universal' PCR primers which are widely available and published in the literature that beginning any new prey detection assay is relatively straightforward without the need for designing new primers (Admassu et al., 2006, Folmer et al., 1994, Pons, 2006, King et al., 2010, Jarman et al., 2006, Vestheim et al., 2005, Zeale et al., 2011).

For more specific target species, PCR primers can be made using a wide range of beneficial software (Rychlik, 2007, Fu et al., 2008, Jarman, 2004, Thornton and Chhandak, 2011) enabling any ecological application of molecular prey detection to be developed and for the results to be quickly and efficiently interpreted as simple presence or absence of prey/species/groups of species. Additionally, the wide range of 'universal' primers which have broad utility for the creation of prey inventories or for getting started with completely unknown predator/prey systems are well suited to nearly any new molecular prey detection experiment (Blankenship and Yayanos, 2005, Pegard et al., 2009, Pineau et al., 2005, Pons, 2006).

High reproducibility and rapid results

The reproducible nature of molecular biology has been greatly increased with the advent of 'kit' based DNA/RNA extraction as well as PCR and qPCR reagents. As a result, greater cross-regional

collaborations are now possible by using identical reagents and sample processing chemistries at numerous facilities. It is also now currently possible to more easily perform assays which have been developed by colleagues from other institutes as is demonstrated here by the publication of numerous group-specific and species-specific PCR primers and the use of previously published primer sets when implementing a new prey detection or species identification assay. A prime driver is the rapid expansion of the international genetics databases which have been populated by researchers from around the world (Hebert et al., 2003b, Ward et al., 2005) who have all used a standard gene region to sequence species in their collections (Hajibabaei et al., 2006). Unlike previous techniques where visual identifications were being performed by differently experienced and trained individuals or teams, the use of genetics databases ensures that all identifications are exactly the same, based upon sequence data.

7.7 Discussion

The diet of marine predators can be hard to determine and even more difficult to quantify accurately because of biases associated with the methods for identifying prey items (Mayfield et al., 2000c, Pierce and Boyle, 1991, Williams, 1981). The most common method for studying predator diet is still the morphological identification of prey items recovered from stomach samples (Joyce et al., 2002, Reñones et al., 2002, Brunnenschweiler et al., 2005, Kamler and Pope, 2001, Phillips et al., 2001, Phillips et al., 2003). Although a common goal of dietary studies is to estimate quantities of food items consumed by populations of a predator, there are numerous biases in prey species identification caused by differential digestion of morphologically identifiable items. This leads to inaccurate estimates of food composition and therefore most (if not all) attempts at dietary quantification have caveats associated with absolute predation quantification (Weber and Lundgren, 2009b, Deagle and Tollit, 2006).

DNA has recently been used as an alternative approach to determining predator diet with a range of ecological and technical questions/applications being resolved by using these techniques including: diverse prey of large predatory fish (Smith et al., 2005), species identification of krill consumed by whale sharks (Jarman and Wilson, 2004) and larval cod in the stomach of predatory fishes (Rosel and Kocher, 2002). DNA has additionally been used to examine prey items with some examples including: species identification of fish consumed by giant squid (Deagle et al., 2005a), salmonids eaten by harbour seals (Purcell et al., 2004) and grey seals (Parsons et al., 2005) and identification of nototheniid fish consumed by Adelie penguins (Jarman et al., 2002).

DNA does have several advantages over conventional morphological analysis as the potential dietary biomarker of choice primarily because DNA is universally present in all food organisms and all food organisms have a unique DNA sequence (Symondson, 2002). Most prey tissues, therefore, contain DNA and because only a small amount of DNA is needed for analysis, DNA is easily detectable even in minute quantities, making it suitable for dietary studies which rely on digested and degraded material (Gagnon et al., 2011, Harper et al., 2005, King et al., 2008a). As the biological function of DNA is to store information there are multiple DNA repositories within every living cell- primarily the nuclear DNA and the mitochondrial DNA (Freeland et al., 2011) and some of the information contained in each DNA molecule can be used to identify individuals, species or higher taxa.

Two short DNA regions unique to a given species or group of species can be identified and PCR primers designed to be specific to these regions can be used to amplify DNA only from this group of organisms. An example of this molecular taxonomic 'group-specific' approach has been used to detect the presence or absence of a wide and varied range of prey items and we have outlined the design and utility of this approach to determine the longevity of the DNA 'signal' in the gut of

rock lobsters in captivity (Chapter 2 and 3). Using additional group-specific PCR primers which effectively amplify prey DNA- avoiding predator DNA and by using a twostep nested PCR-based approach, it has been demonstrated for the first time that prey items can be identified from *Octopus vulgaris* paralarvae collected in the wild (Roura et al., 2012). A range of ecological questions and aquaculture experiments can now be addressed concerning the trophic role of *Octopus vulgaris* and the potential for rearing of this species in order to increase the low survival of this early life stage, a goal which has been actively pursued for over 50 years (Itami et al., 1963, Ambrose, 1984, Wells, 1978). The more focused 'species-specific' PCR based approach to dietary analysis was explored in Chapter 5, where the relationship between rock lobsters and two species of sea urchins is examined with *in situ* field experiments and molecular prey detection techniques. The use of a directed 'species-specific' molecular assay illustrates the potential to answer ecological questions using DNA-based tools and highlights the benefits of using DNA as a dietary biomarker.

The species level identification of discrete prey remains can be a major advantage of using DNA to resolve predator diet. The example of the seven gilled shark (Barnett et al., 2009) illustrates this point clearly, as the initial visual inspection of stomach contents could reliably identify only 42% of the prey remains, but by sequencing the DNA from the unidentified items, the species level identifications went up to 85%. Of the 22 prey species found to comprise the diet of the seven gilled shark, 7 species were only identified by using DNA sequencing technology (Barnett et al., 2009). This demonstrates an additional benefit of using molecular techniques for the improvement of taxonomic information available from degraded dietary samples.

Further detail about the complete inventory of potential prey species within a mixed dietary sample may be obtained either by cloning and sequencing or with gel mobility methods, the latter of which was not explored in this thesis (Martin et al., 2006, Diez et al., 2001, Deagle et al., 2005a). The broad dietary information which can be gained by DNA cloning and sequencing ('clone library') analysis is described in Chapter 6.

Molecular methods have certainly proven to be the most effective way to determine the diet of *Octopus vulgaris* paralarvae due to its pelagic habitat, small size, ingestion mechanism and high digestion rates, which make traditional detection of prey with morphological analyses impossible (Roura et al., 2012, Roura et al., 2010). This is the first time that prey items have been identified in *Octopus vulgaris* paralarvae taken from the wild. In a captive study, the first step was to develop a molecular technique to detect prey inside a single paralarvae with species-specific primers (Roura et al., 2010). This new approach represented the next logical step, aimed at understanding the trophic ecology which has been in question since the first attempt to culture the species (Itami et al., 1963). These results can be directly applied to aquaculture in order to better understand the nutritional requirements of larvae in the wild which is expected to assist in improving the low survival rate that octopus display in captivity (Villanueva and Norman, 2008). Besides the industrial application of this data and technical development, the techniques have begun to unravel a key component of the ecology of *Octopus vulgaris* paralarvae within the complex pelagic food web.

The initial challenges of amplifying DNA from crustaceans with universal primers hindered the creation of the genetic database of the prey species present in the Ría of Vigo. It was therefore necessary to perform this prey inventory with the primer set 16Sar-16Sscr. Nevertheless, copepods had to be excluded from the database because we could only amplify prey DNA from

them when removed from the *Octopus vulgaris* template. Although the designed group-specific primers only differed in 4 nucleotides with aligned copepods *in silico*, this variation was sufficient to preclude copepod amplifications at all possible annealing temperatures. This unexpected failure to amplify copepod DNA is a potential consequence of using group-specific primers (Jarman et al., 2004, Braley et al., 2010, Deagle et al., 2005b) which have been designed to exclude from amplification *Octopus vulgaris* DNA. Nevertheless, an interesting application of this primer affinity demonstrates that these primers can be used to identify prey in copepods with the obvious benefit of excluding the predator.

It was remarkable that three of the four prey species identified in copepods were also detected in *Octopus vulgaris* gut content clones. In fact, six copepods matched 80-82% with *Alpheus distinguendus*, a prey that was recovered in 20 clones from *Octopus vulgaris* and *Pirimela denticulata* which was detected in 3 copepods (100%), which also corresponded to 4 of the clones obtained from *Octopus vulgaris* gut contents. Whether this represents a detection of target prey or a case of secondary predation, is uncertain.

A group specific reverse primer designed for crustaceans (Braley et al., 2010) was tested in conjunction with 16Sar (Simon et al. 1994) but this pair did not discriminate between predator and prey DNA. This inefficacy has been previously described (Simon et al 1994) as only 11 of 184 PCR attempts produced successful amplifications of krill and shrimp with the crustacean-specific primer. Another universal primer pair with degenerate sequences designed by Deagle et al. (2007) was tested but failed to generate the anticipated amplicons from *Octopus vulgaris* paralarvae gut samples. It is important to note that those degenerate group specific primers were designed based on primers used in Deagle et al. (2005), which targeted cephalopods and teleost fishes. The universal degenerate primers designed in Deagle et al. (2007) exclusively amplified DNA from fishes (59 clones) and cephalopod (1 clone). When applied to our *Octopus vulgaris* paralarvae gut

samples, the only result was a continuous smear in all samples tested (crustaceans, chaetognats and octopus, data not shown) under a wide range of different annealing conditions.

At several stages of analysis, species or genus level identifications were not possible because no similar sequences were present in either the GenBank or Barcode of Life databases. Phylogenetic relatedness was, in these cases, indispensable as it was necessary to assign the unidentified sequences to the highest/closest taxonomic lineage possible. This reflects the difficulty when working with the diet of generalist predators where there is limited sequence information available to target the large diversity of potential prey taxa (Blankenship and Yayanos, 2005, Suzuki et al., 2006). A well-known prerequisite for resolving the diet of any generalist predator is the extensive characterization of the system (King et al., 2008a). In this work all the sequences that were submitted to GenBank from zooplankton species (4) were also detected in the gut of the paralarvae (Roura et al., 2010, Roura et al., 2012). This highlights the importance of an appropriate genetic database to obtain the highest level of identification and to reduce the uncertainty of any species identifications. More efforts should be undertaken to increase the comprehensive nature of the genetic databases for marine organisms which will increase the taxonomic level of identifications at the genera or species level.

7.8 Conclusion and future directions

The primary aim of this work was to develop molecular methods to identify the natural prey of several important temperate marine species. This dietary knowledge is critical to understanding trophic links and to building models for managing marine resources. There are additional benefits which will aid in the design of an optimal diet for the planktonic larval phases for potential aquaculture operations and for understanding the implications of large predators in nursery systems such as shark nursery areas. In conclusion, several PCR-based techniques have

been successfully created, forming the basis of a suite of novel molecular analyses to detect prey in a wide range of marine predators obtained from the wild. Several of the model systems explored were the first attempt to unravel the complex trophic interactions for these species and at present, it is unclear whether these data are just scratching the surface with these dietary studies or if the full spectrum of prey consumed have been completely resolved, but undoubtedly this initial step is an important starting point to addressing fundamental questions about the ecology of these animals. All molecular techniques have particular limitations, and therefore studying the diet of any generalist predator ideally requires a combined approach to fully understand its biology.

The next directions and applications of these techniques should revolve around the variation of predator diet across broad spatial and temporal scales. Of particular interest is the impact of climate change on trophic interactions in ecosystems. As waters warm, species are moving pole wards but at different rates. New ecosystems and ecosystem interactions are evolving. There is also considerable scope to expand the research around the differences in diet between fished and unfished locations to augment or reduce the need for traditional manipulative experiments (caging, exclusions, removals, etc.) which ultimately have an impact on marine ecosystems. It is also recommended that these molecular techniques are expanded into bioregions where extensive predator/prey research is already underway. Many authors in these areas (New Zealand and South Africa in particular) strive to demonstrate trophic relationships in complex temperate reef systems and have shown that rock lobsters, in particular are indeed a top predator exerting top down regulation. The debates about fishing quotas, annual harvest limits, upper/lower size limits and spatial closures need as much additional information as possible to better inform marine resource managers. The work in this thesis represents another important

tool for understanding the complex nature of marine ecosystems and a way forward for the next generation of marine ecologists.

Chapter 8

Conclusion: Resolving marine predator diet on the molecular frontier

“...Have to admit, I felt a little out of place,

But I swear to God, I have seen it all now,

Nothing shocks me anymore....”

Seen it all

Jake Bugg

Jake Bugg

2013

Chapter 8: The conclusion: resolving marine predator diet on the molecular frontier

8.1 Introduction

I show clearly that molecular techniques can be used successfully to detect specific prey consumed by the southern lobster (*Jasus edwardsii* Family Palinuridae) *in situ* and to this end, have developed a suite of sensitive and ecologically important assays to fit this species. I have also explored the diet of a range of other important, but poorly understood, temperate marine predators including the common octopus (*Octopus vulgaris*) and the seven gilled shark (*Notorynchus cepedianus*).

In most cases an efficient and non-lethal method was used to collect dietary samples and this approach, although necessitating additional and careful field handling of the animals, has provided a platform for the deployment of similar assays on endangered / rare / threatened species and for work in protected marine areas (Chapters 2 and 7).

Strengths and weaknesses of DNA as a dietary biomarker need to be understood for any new dietary study. Species-specific DNA-based approaches are not intended for: 'What does animal X eat' type studies, but will better suit broad temporal, spatial and individual level diet estimation questions. Species-specific DNA-based approaches are particularly good for directed questions and for obtaining high taxonomic resolution when studying predation *in situ*. The case study we present for exploring the relationship between rock lobsters, sea urchins and temperate reef structure (Chapter 5) is an example of using this approach on an ecologically relevant scale. As such, this thesis represents a milestone in the field of molecular trophic interactions and demonstrates the utility of using DNA-based techniques on a large scale.

Presently the technology for DNA quantification is excellent but interpretation of this data in the dietary context remains a considerable challenge. There are not many technical difficulties in exploiting the potential of DNA for animal diet estimation but validating these studies from wild samples is particularly problematic. The quantitative PCR approach can determine the number of copies of prey DNA present in a sample, but is currently unable to discern the difference between a small amount of prey consumed recently and a larger amount consumed a longer time in the past which has remained after extended digestion by the predator (Chapter 4).

8.2 Conclusion

The primary aim of this work was to develop molecular methods to identify the natural prey of several important marine species. This dietary knowledge is critical to understanding trophic links and to building models for managing marine resources. There are additional benefits which will aid in the design of an optimal diet for the planktonic larval phases for potential aquaculture operations and for understanding the implications of large predators in nursery systems such as shark nursery areas. In conclusion, I have successfully created several PCR-based techniques which form the basis of a suite of novel molecular analyses to detect prey in a wide range of marine predators obtained from the wild. Several of the model systems I explored were the first attempt to unravel the complex trophic interactions for these species and I am presently unsure of whether we are just scratching the surface with these dietary studies or we have almost completely resolved the full spectrum of prey consumed, but undoubtedly this initial step is an important starting point to addressing fundamental questions about the ecology of these animals. All molecular techniques have particular limitations, and therefore studying the diet of any generalist predator ideally requires a combined approach to deeply understand its biology.

The next directions and applications of these techniques should revolve around investigating the variation of predator diet across broad spatial and temporal scales. Of particular interest is the impact of climate change on trophic interactions in ecosystems. As waters warm, species are moving polewards but at different rates. New ecosystems and ecosystem interactions are evolving. There is also considerable scope to expand the research around the differences in diet between fished and unfished locations to augment or reduce the need for traditional

manipulative experiments (caging, exclusions, removals, etc.) which ultimately have an impact on marine ecosystems. It is also recommended that these molecular techniques are expanded into bioregions where extensive predator/prey research is already underway. Many authors in these areas (New Zealand and South Africa in particular) strive to demonstrate trophic relationships in complex temperate reef systems and have shown that rock lobsters, in particular, are indeed a top predator exerting top down regulation. The debates about fishing quotas, annual harvest limits, upper/lower size limits and spatial closures need as much additional information as possible to better inform marine resource managers. The work in this thesis represents another important tool for understanding the complex nature of marine ecosystems and a way forward for the next generation of marine ecologists.

References

- ADMASSU, B., JUEN, A. & TRAUGOTT, M. 2006. Earthworm primers for DNA-based gut content analysis and their cross- reactivity in a multi-species system. *Soil Biology and Biochemistry*, 38, 1308-1315.
- AGUSTI, N., DE VICENTE, M. & GABARRA, R. 1999. Development of sequence amplified characterized region (SCAR) markers of *Helicoverpa armigera*: a new polymerase chain reaction-based technique for predator gut analysis. *Molecular Ecology*, 8, 1467-1474.
- AGUSTI, N., SHAYLER, S., HARWOOD, J., VAUGHAN, I., SUNDERLAND, K. & SYMONDSON, W. 2003a. Collembola as alternative prey sustaining spiders in arable ecosystems: prey detection within predators using molecular markers. *Molecular Ecology*, 12, 3467-3475.
- AGUSTI, N., UNRUH, T. & WELTER, S. 2003b. Detecting *Cacopsylla pyricola* (Hemiptera: Psyllidae) in predator guts using COI mitochondrial markers. *Bulletin of Entomological Research*, 93, 179-185.
- ALLCOCK, A., COOKE, I. & STRUGNELL, J. 2011. What can the mitochondrial genome reveal about higher-level phylogeny of the molluscan class Cephalopoda? *Zoological Journal of the Linnean Society*, 161, 573–586.
- ALTSHULER, M. 2006. *PCR troubleshooting : the essential guide*, Wymondham, Caister Academic Press.
- AMBROSE, R. 1984. Food preferences, prey availability and the diet of *Octopus bimaculatus* *Journal of Experimental Marine Biology and Ecology*, 77, 29-44.
- ANDREW, N. & MACDIARMID, A. 1991. Interrelations between sea urchins and spiny lobsters in northern New Zealand. *Marine Ecology Progress Series*, 70, 211-222.
- ANDREW, N. & O'NEILL, A. 2000. Large-scale patterns in habitat structure on subtidal rocky reefs in New South Wales. *Marine & Freshwater Research*, 51, 255-263.
- ARVIDSSON, S., KWASNIEWSKI, M., RIAÑO-PACHÓN, D. & MUELLER-ROEBER, B. 2008. QuantPrime - A flexible tool for reliable high-throughput primer design for quantitative PCR. *BMC Bioinformatics*, 9, 465.
- BABCOCK, R., SHEARS, N., ALCALA, A., BARRETT, N., EDGAR, G., LAFFERTY, K., MCCLANAHAN, T. & RUSS, G. 2010a. Decadal trends in marine reserves reveal differential rates of change in direct and indirect effects. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 18256-18261.
- BABCOCK, R., SHEARS, N., ALCALAC, A., BARRETT, N., EDGARD, G., LAFFERTYE, K., MCCLANAHAN, T. & RUSS, G. 2010b. Decadal trends in marine reserves reveal differential rates of change in direct and indirect effects. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 18256-18261.
- BARKAI, A., DAVIS, C. & TUGWELL, S. 1996. Prey selection by the South African cape rock lobster *Jasus lalandii*: Ecological and physiological approaches. *Bulletin of Marine Science*, 58, 1-8.
- BARNETT, A., REDD, K., FRUSHER, S., STEVENS, J. & SEMMENS, J. 2009. Non- lethal method to obtain stomach samples from a large marine predator and the use of DNA analysis to improve dietary information. *Journal of Experimental Marine Biology and Ecology* 393, 188-192.
- BARNETT, A., REDD, K., FRUSHER, S., STEVENS, J. & SEMMENS, J. 2010a. Non- lethal method to obtain stomach samples from a large marine predator and the use of DNA analysis to improve dietary information. *Journal of Experimental Marine Biology and Ecology* 393, 188-192.
- BARNETT, A., REDD, K., FRUSHER, S., STEVENS, J. & SEMMENS, J. 2010b. Non- lethal method to obtain stomach samples from a large marine predator and the use of DNA analysis to improve dietary information. . *Journal of Experimental Marine Biology and Ecology*, 393, 188-192.

- BARRETT, N., EDGAR, G., MORTON, A. & BUXTON, C. 2003. A decade of study in Tasmanian MPAs- Part 2. *Fishing Today*, 16, 21-23.
- BECKERMAN, A., PETCHEY, O. & WARREN, P. 2006. Foraging biology predicts food web complexity *Proceedings of the National Academy of Sciences of the United States of America*, 103, 13745-13749
- BENSON, D., KARSCH-MIZRACHI, I., LIPMAN, D., OSTELL, J. & WHEELER, D. 2008. GenBank. *Nucleic Acids Research 2008 (Database issue)*, 36, 25-30.
- BLAMEY, L., BRANCH, G. & REAUGH-FLOWER, K. 2010. Temporal changes in kelp forest benthic communities following an invasion by the rock lobster *Jasus lalandii*. *African Journal of Marine Science*, 32, 481-490.
- BLANKENSHIP, L. & YAYANOS, A. 2005. Universal primers and PCR of gut contents to study marine invertebrate diets. *Molecular Ecology*, 14, 891-899.
- BLAXTER, M., MANN, J., CHAPMAN, T., THOMAS, F., WHITTON, C., FLOYD, R. & ABEBE, E. 2005. Defining operational taxonomic units using DNA barcode data. *Philosophical Transactions of the Royal Society Biological Sciences*, 360, 1935-1943.
- BOWLES, E., SCHULTE, P., TOLLIT, D., DEAGLE, B. & TRITES, A. 2011. Proportion of prey consumed can be determined from faecal DNA using real-time PCR. *Molecular Ecology Resources*, In press.
- BOWMAN, J. & MCCUAIG, R. 2003. Biodiversity, Community Structural Shifts, and Biogeography of Prokaryotes within Antarctic Continental Shelf Sediment. *Applied and Environmental Microbiology*, 69, 2463-2483.
- BRACCINI, J. 2008. Feeding ecology of two high-order predators from south-eastern Australia: the coastal broadnose and the deepwater sharpnose sevengill sharks. *Marine Ecology Progress Series*, 371, 273-284.
- BRALEY, M., GOLDSWORTHY, S., PAGE, B., STEER, M. & AUSTIN, J. 2010. Assessing morphological and DNA-based diet analysis techniques in a generalist predator, the arrow squid *Nototodarus gouldi*. *Molecular Ecology Resources*, 10, 466-474.
- BREEN, P. & MANN, K. 1976. Changing lobster abundance and the destruction of kelp beds by sea urchins. *Marine Biology*, 34, 137-142.
- BROCK, D. & WARD, T. 2004. Maori octopus (*Octopus maorum*) bycatch and southern rock lobster (*Jasus edwardsii*) mortality in the South Australian lobster fishery. *Fishery Bulletin*, 102, 430-440.
- BROWN, D., JARMAN, S. & SYMONDSON, W. 2012. Pyrosequencing of prey DNA in reptile faeces: analysis of earthworm consumption by slow worms. *Molecular Ecology Resources*, 12, 259-266.
- BROWN, M. & BOWMAN, J. 2001. A molecular phylogenetic survey of sea-ice microbial communities. *FEMS Microbiology Ecology*, 35, 267-275.
- BRUNNSCHWEILER, J., ANDREWS, P., SOUTHALL, E., PICKERING, M. & SIMS, D. 2005. Rapid voluntary stomach eversion in a free-living shark. *Journal of the Marine Biological Association of the United Kingdom*, 85, 1141-1144.
- BUSTIN, S. (ed.) 2004. *A-Z of quantitative PCR*, La Jolla, CA: International University Line.
- BUTLER, I., MARK, J. & KINTZING, M. 2016. An exception to the rule: top-down control of a coral reef macroinvertebrate community by a tropical spiny lobster. *Bulletin of Marine Science*, 92, 137-152.
- CALLAWAY, T., DOWD, S., EDRINGTON, T., ANDERSON, R., KRUEGER, N., BAUER, N., KONONOFF, P. & NISBET, D. 2010. Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing *Journal of Animal Science*, 88, 3977 - 3983.
- CASPER, R., JARMAN, S., DEAGLE, B., GALES, N. & HINDELL, M. 2007a. Detecting prey from DNA in predator scats: A comparison with morphological analysis, using *Arctocephalus* seals fed a known diet *Journal of Experimental Marine Biology and Ecology* 347, 144-154

- CASPER, R., JARMAN, S., GALES, N. & HINDELL, M. 2007b. Combining DNA and morphological analyses of faecal samples improves insight into trophic interactions: a case study using a generalist predator. *Marine Biology*, 152, 815-825.
- CHEN, B., JANES, H. & CHEN, S. 2002. Computer programs for PCR primer design and analysis. *Methods in molecular biology*, 192, 19-29.
- CHEN, Y., GILES, L., PAYTON, E. & GREENSTONE, H. 2000. Identifying key cereal aphid predators by molecular gut analysis. *Molecular Ecology*, 9, 1887-1898.
- CHOW, S., SUZUKI, N., IMAI, H. & YOSHIMURA, T. 2006. Molecular species identification of spiny lobster phyllosoma larvae of the genus *Panulirus* from the northwestern Pacific. *Marine Biotechnology*, 8, 260-267.
- CHOW, S., SUZUKI, S., MATSUNAGA, T., LAVERY, S., JEFFS, A. & TAKEYAMA, H. 2011. Investigation on Natural Diets of Larval Marine Animals Using Peptide Nucleic Acid-Directed Polymerase Chain Reaction Clamping. *Marine Biotechnology*, In press, 1-9.
- CHRISTOFOLETTI, R., MURAKAMI, V., OLIVEIRA, D., BARRETO, R. & FLORES, A. 2010. Foraging by the omnivorous crab *Pachygrapsus transversus* affects the structure of assemblages on sub-tropical rocky shores. *Marine Ecology Progress Series*, 420, 125-134.
- CLARE, E., BARBER, B., SWEENEY, B., HEBERT, P. & FENTON, M. 2011. Eating local: influences of habitat on the diet of little brown bats (*Myotis lucifugus*). *Molecular Ecology*, 20, 1772-1780.
- CLARE, E., FRASER, E., BRAID, H., FENTON, M. & HEBERT, P. 2009 Species on the menu of a generalist predator, the eastern red bat (*Lasiurus borealis*): using a molecular approach to detect arthropod prey. *Molecular Ecology*, 18, 2532-42.
- CLARE, E. L. 2014. Molecular detection of trophic interactions: emerging trends, distinct advantages, significant considerations and conservation applications. *Evolutionary Applications*, 7, 1144-1157.
- CORRIGAN, L., WINFIELD, I., HOELZEL, A. & LUCAS, M. 2011. Dietary plasticity in Arctic charr (*Salvelinus alpinus*) in response to long-term environmental change *Ecology of Freshwater Fish*, 20, 5-13
- CORSE, E., VALLADARES, S., PLANAS, M., CHAMORRO, A. & PINTADO, J. 2015. Analysis of the diet of the long-snouted seahorse *Hippocampus guttulatus* by 18SrDNA amplification of prey in faeces. *Aquaculture Nutrition*, 21, 528-540.
- CORTES, E. & GRUBER, S. 1990. Diet, feeding habits and estimates of daily ration of young lemon sharks, *Negaprion brevirostris* (Poey). *Copeia*, 1, 204-218.
- COWEN, R. 1983. The effects of sheephead (*Semicossyphus pulcher*) predation on red sea urchin (*Strongylocentrotus franciscanus*) populations: an experimental analysis. *Oecologia*, 58, 249-255.
- COX, C., HUNT, J., LYONS, W. & DAVIS, G. 1997. Nocturnal foraging of the Caribbean spiny lobster (*Panulirus argus*) on offshore reefs of Florida, USA. *Marine and Freshwater Research*, 48, 671 - 680
- COYLE, K., EISNER, L., MUETER, F., PINCHUK, A., JANOUT, M., CIECIEL, K., FARLEY, E. & ANDREWS, A. 2011. Climate change in the southeastern Bering Sea: Impacts on pollock stocks and implications for the oscillating control hypothesis *Fisheries Oceanography*, 20, 139-156
- DALÉN, L., GÖTHERSTRÖM, A. & ANGERBJÖRN, A. 2004. Identifying species from pieces of faeces. *Conservation Genetics*, 5, 109-111.
- DEAGLE, B., CHIARADIA, A., MCINNES, J. & JARMAN, S. 2010. Pyrosequencing faecal DNA to determine diet of little penguins: is what goes in what comes out? *Conservation Genetics*, 11, 2039-2048
- DEAGLE, B., EVESON, J. & JARMAN, S. 2006. Quantification of damage in DNA recovered from highly degraded samples-a case study on DNA in faeces. *Frontiers in Zoology*, 3.

- DEAGLE, B., GALES, N., K, E., JARMAN, S., ROBINSON, S., TREBILCO, R. & HINDELL, M. 2007. Studying seabird diet through genetic analysis of faeces: a case study on macaroni penguins (*Eudyptes chrysolophus*). *PLoS ONE*, 2, e831.
- DEAGLE, B., JARMAN, S., PEMBERTON, D. & GALES, N. 2005a. Genetic Screening for Prey in the Gut Contents from a Giant Squid (*Architeuthis sp.*). *Journal of Heredity*, 96, 417-423.
- DEAGLE, B., KIRKWOOD, R. & JARMAN, S. 2009. Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. *Molecular Ecology*, 18, 2022–2038.
- DEAGLE, B. & TOLLIT, D. 2006. Quantitative analysis of prey DNA in pinniped faeces: potential to estimate diet composition? *Conservation Genetics*, 10.1007/s10592-006-9197-7.
- DEAGLE, B., TOLLIT, D., JARMAN, S., HINDELL, M., TRITES, A. & GALES, N. 2005b. Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. *Molecular Ecology*, 14, 1831-1842.
- DENNY, B. 2010. *RE: Rock lobster foraging behaviour and activity patterns*. Type to LING, S.
- DIEZ, B., PEDROS-ALIO, C., MARSH, T. & MASSANA, R. 2001. Application of Denaturing Gradient Gel Electrophoresis (DGGE) To Study the Diversity of Marine Picoeukaryotic Assemblages and Comparison of DGGE with Other Molecular Techniques. *Applied and Environmental Microbiology*, 67, 2942-2951.
- DIGGLE, M. & CLARKE, S. 2004. PyrosequencingTM: Sequence typing at the speed of light. *Molecular Biotechnology*, 28, 129-137.
- DORIGO, U., BERARD, A. & HUMBERT, J. 2002. Comparison of Eukaryotic Phytobenthic Community Composition in a Polluted River by Partial 18S rRNA Gene Cloning and Sequencing. *Microbial Ecology*, 44, 372-380.
- DUNSHEA, G. 2009. DNA-Based diet analysis for any predator. *PLoS ONE*, 4, e5252.
- EDGAR, G. & BARRETT, N. 1999. Effects of the declaration of marine reserves on Tasmanian reef fishes, invertebrates and plants. *Journal of Experimental Marine Biology and Ecology*, 242, 107-144.
- EDGAR, G., BARRETT, N. & STUART-SMITH, R. 2009. Exploited reefs protected from fishing transform over decades into conservation features otherwise absent from seascapes. *Ecological Applications*, 19, 1967–1974.
- EDGAR, G., MOVERLEY, J., BARRETT, N., PETERS, D. & REED, C. 1997. The conservation-related benefits of a systematic marine biological sampling programme: The Tasmanian reef bioregionalisation as a case study. *Biological Conservation*, 79, 227-240.
- EDWARDS, R., RODRIQUEZ-BRITO, B., WEGLEY, L., HAYNES, M., BRIETBART, M., PETERSON, D., SAAR, M., ALEXANDER, S., ALEXANDER, C. & ROHWER, F. 2006. Using pyrosequencing to shed light on deep mine microbial ecology. *BMC Genomics*, 7.
- ELLIOTT, N., BARTLETT, J., EVANS, B., OFFICER, R. & HADDON, M. 2002. Application of molecular genetics to the Australian abalone fisheries: forensic protocols for species identification and blacklip stock structure. Hobart, Tasmania: CSIRO Marine Research and Fisheries Research and Development Corporation.
- ENNIS, G. 1973. Food, feeding, and condition of lobsters, *Homarus americanus*, throughout the seasonal cycle in Bonavista Bay, Newfoundland. *Journal of the Fisheries Research Board of Canada*, 30, 1905-1909.
- ESTES, J. & DUGGINS, D. 1995. Sea otters and kelp forests in Alaska: generality and variation in a community ecological paradigm. *Ecological Monographs*, 65, 75–100.
- ESTES, J. & PALMISANO, J. 1974a. Sea otters: their role in structuring nearshore communities. *Science*, 185, 1058-1060.
- ESTES, J. & PALMISANO, J. 1974b. Sea otters: their role in structuring nearshore communities. *Science*, 185, 1058-1060.
- ESTES, J., SMITH, N. & PALMISANO, J. 1978. Sea Otter Predation and Community Organization in the Western Aleutian Islands, Alaska. *Ecology*, 59, 822-833.

- ESTES, J., TINKER, M., WILLIAMS, T. & DOAK, D. 1998. Killer whale predation on sea otters linking oceanic and nearshore ecosystems. *Science*, 282, 473-476.
- FARRELL, E., ROMAN, J. & SUNQUIST, E. 2000. Dietary separation of sympatric carnivores identified by molecular analysis of scats. *Molecular Ecology*, 9, 1583-1590.
- FIELDER, D. 1965. The Spiny Lobster, *Jasus lalandei* (H. Milne Edwards), in South Australia: Food, Feeding, and Locomotor Activity. *Australian Journal of Marine and Freshwater Research*, 16, 351-367.
- FOLLESA, M. C., PORCU, C., GASTONI, A., MULAS, A., SABATINI, A. & CAU, A. 2009. Community structure of bathyal decapod crustaceans off South-Eastern Sardinian deep-waters (Central-Western Mediterranean). *Marine Ecology-an Evolutionary Perspective*, 30, 188-199.
- FOLMER, O., BLACK, M., HOEH, W., LUTZ, R. & VRIJENHOEK, R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3, 294-299.
- FOLTAN, P., SHEPPARD, S., KONVICKA, M. & SYMONDSON, W. 2005. The significance of facultative scavenging in generalist predator nutrition: detecting decayed prey in the guts of predators using PCR. *Molecular ecology*, 14, 4147-4158.
- FORAN, D. 2008. Review of Forensic DNA Analysis: A Laboratory Manual. *Journal of Forensic Sciences*, 53, 1238.
- FOSTER, M. & SCHIEL, D. 2010. Loss of predators and the collapse of southern California kelp forests (?): Alternatives, explanations and generalizations. *Journal of Experimental Marine Biology and Ecology*, 393, 59-70.
- FOX, C., TAYLOR, M., VAN DER KOOIJ, J., TAYLOR, N., MILLIGAN, S., ALBAINA, A., PASCOAL, S., LALLIAS, D., MAILLARD, M. & HUNTER, E. 2012. Identification of marine fish egg predators using molecular probes. *Marine Ecology Progress Series*, 462, 205-218.
- FREELAND, J., PETERSEN, S. & KIRK, H. 2011. *Molecular Ecology*, Wiley-Blackwell.
- FU, Q., RUEGGER, P., BENT, E., CHROBAK, M. & BORNEMAN, J. 2008. PRISE (PRImer SElector): Software for designing sequence-selective PCR primers. *Journal of Microbiological Methods*, 72, 263-267.
- GAGNON, A., DOYON, J., HEIMPEL, G. & BRODEUR, J. 2011. Prey DNA detection success following digestion by intraguild predators: influence of prey and predator species. *Molecular Ecology Resources*, 11, 1022 - 1032.
- GHARIZADEH, B., AKHRAS, M., NOURIZAD, N., GHADERI, M., YASUDA, K., NYREN, P. & POURMAND, N. 2006. Methodological improvements of pyrosequencing technology *Journal of Biotechnology*, 124, 504-511.
- GÓES, C. & LINS-OLIVEIRA, J. 2009. Natural diet of the spiny lobster, *Panulirus echinatus* Smith, 1869 (Crustacea: Decapoda: Palinuridae), from São Pedro and São Paulo Archipelago, Brazil. *Brazilian Journal of Biology*, 69, 143-148.
- GREEN, S. & MINZ, D. 2005. Suicide polymerase endonuclease restriction, a novel technique for enhancing PCR amplification of minor DNA templates. *Applied and Environmental Microbiology*, 71, 4721-4727.
- GROENEVELD, J. C., MAHARAJ, G. & SMITH, C. D. 2006. *Octopus magnificus* predation and bycatch in the trap fishery for spiny lobsters *Palinurus gilchristi* off South Africa. *Fisheries Research*, 79, 90-96.
- GUEST, M., FRUSHER, S., NICHOLS, P., JOHNSON, C. & WHEATLEY, K. 2009. Trophic effects of fishing southern rock lobster *Jasus edwardsii* shown by combined fatty acid and stable isotope analyses. *Marine Ecology Progress Series*, 388, 169-184.
- GUIDETTI, P. 2006 Marine Reserves Reestablish Lost Predatory Interactions And Cause Community Changes In Rocky Reefs. *Ecological Applications*, 16, 963-976.
- HAARR, M. L. & ROCHETTE, R. 2012. The effect of geographic origin on interactions between adult invasive green crabs *Carcinus maenas* and juvenile American lobsters *Homarus*

- americanus* in Atlantic Canada. *Journal of Experimental Marine Biology and Ecology*, 422, 88-100.
- HAGEN, N. T. & MANN, K. H. 1992. Functional-response of the predators American lobster *Homarus americanus* (Milne-Edwards) and Atlantic wolffish *Anarhichas lupus* (L) to increasing numbers of the green sea-urchin *Strongylocentrotus droebachiensis* (Muller). *Journal of Experimental Marine Biology and Ecology*, 159, 89-112.
- HAJIBABAEI, M., SMITH, A., JANZEN, D., RODRIGUEZ, J., WHITFIELD, J. & HEBERT, P. 2006. A minimalist barcode can identify a specimen whose DNA is degraded. *Molecular Ecology Notes*.
- HAKALA, J. & JOHNSON, F. 2004. Evaluation of a Gastric Lavage Method for Use on Largemouth Bass. *North American Journal of Fisheries Management*, 24, 1398-1403.
- HALEY, C., BLAMEY, L., ATKINSON, L. & BRANCH, G. 2011. Dietary change of the rock lobster *Jasus lalandii* after an 'invasive' geographic shift: Effects of size, density and food availability. *Estuarine, Coastal and Shelf Science*, 93, 160-170.
- HALPERN, B. & WARNER, R. 2002. Marine reserves have rapid and lasting effects. *Ecology Letters*, 5, 361-366.
- HANSON, J. M. 2009. Predator-prey interactions of American lobster (*Homarus americanus*) in the southern Gulf of St. Lawrence, Canada. *New Zealand Journal of Marine and Freshwater Research*, 43, 69-88.
- HANSON, J. M., COMEAU, M. & RONDEAU, A. 2014. Atlantic Rock Crab, unlike American Lobster, Is Important to Ecosystem Functioning in Northumberland Strait. *Transactions of the American Fisheries Society*, 143, 1266-1279.
- HARPER, G., KING, R., DODD, C., HARWOOD, J., GLEN, D., BRUFORD, M. & SYMONDSON, W. 2005. Rapid screening of invertebrate predators for multiple prey DNA targets. *Molecular Ecology*, 14, 819-827.
- HARRINGTON, J., SEMMENS, J., GARDNER, C. & FRUSHER, S. 2006. Predation of trap-caught southern rock lobsters, *Jasus edwardsii* (Hutton, 1875), in Tasmanian waters by the Maori octopus, *Octopus maorum* (Hutton, 1880): Spatial and temporal trends. *Fisheries Research*, 77, 10-16.
- HEBERT, P., CYWINSKA, A., BALL, S. & DEWAARD, J. 2003a. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London*, 270, 313-322.
- HEBERT, P., CYWINSKA, A., S.L., B. & DEWAARD, J. 2003b. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London*, 270, 313-322.
- HEBERT, P. & GREGORY, T. 2005. The promise of DNA barcoding for taxonomy. *Systematic Biology*, 54, 852 - 859
- HICKMAN, V. 1945. Notes on the Tasmanian Crayfish, *Jasus lalandii* (Milne Edwards). *Papers and Proceedings Royal Society of Tasmania*, 27-38.
- HOLMES, B., STEINKE, D. & WARD, R. 2008. Identification of shark and ray fins using DNA barcoding. *Fisheries Research*, 95, 280 - 288.
- HOOGENDOORN, M. & HEIMPEL, G. 2001. PCR-based gut content analysis of insect predators: using ribosomal ITS-1 fragments from prey to estimate predation frequency. *Molecular Ecology*, 10, 2059-2067.
- HOYT, M., FLEEGER, J., SIEBELING, R. & FELLER, R. 2000. Serological estimation of prey-protein gut-residence time and quantification of meal size for grass shrimp consuming meiofaunal copepods. *Journal of Experimental Marine Biology and Ecology*, 248, 1-129.
- HUDSON, I. R. & WIGHAM, B. D. 2003. *In situ* observations of predatory feeding behaviour of the galatheid squat lobster *Munida sarsi* using a remotely operated vehicle. *Journal of the Marine Biological Association of the United Kingdom*, 83, 463-464.
- HUGHES, J., HELLMANN, J., RICKETTS, T. & BOHANNAN, B. 2001. Counting the Uncountable: Statistical Approaches to Estimating Microbial Diversity. *Applied and Environmental Microbiology*, 67, 4399-4406.

- HUSE, S., HUBER, J., MORRISON, H., SOGIN, M. & WELCH, D. 2007. Accuracy and quality of massively parallel DNA pyrosequencing. *Genome Biology*, 8.
- HYSLOP, E. 1980. Stomach contents analysis-a review of methods and their application. *Journal of Fisheries Biology*, 17, 411-429.
- INOUE, N., WATANABE, H., KOJIMA, S. & SEKIGUCHI, H. 2007. Population structure of Japanese spiny lobster *Panulirus japonicus* inferred by nucleotide sequence analysis of mitochondrial COI gene. *Fisheries Science*, 73, 550-556.
- IRVIN, S. & TABRETT, S. 2005. A novel method of collecting fecal samples from spiny lobsters. *Aquaculture*, 243, 269-272.
- ITAMI, K., IZAWA, Y., MAEDA, S. & NAKA, I. K. 1963. Notes on the laboratory culture of the octopus larvae. *Bulletin of the Japanese Society of Scientific Fisheries*, 29, 514-520.
- JAEGER, A. & CHEREL, Y. 2011. Isotopic investigation of contemporary and historic changes in penguin trophic niches and carrying capacity of the Southern Indian Ocean. *PLoS ONE*, 6, 16484.
- JARMAN, S. 2004. AMPLICON: Software for Designing PCR Primers on Aligned DNA Sequences. *Bioinformatics*, 20, 1644-1645.
- JARMAN, S. 2006. Cleaver: software for identifying taxon specific restriction endonuclease recognition sites. *Bioinformatics*, 22, 2160 - 2161.
- JARMAN, S., DEAGLE, B. & GALES, N. 2004. Group-specific polymerase chain reaction for DNA-based analysis of species diversity and identity in dietary samples. *Molecular Ecology*, 13, 1313-1322.
- JARMAN, S., GALES, N., TIERNEY, M., GILL, P. & ELLIOTT, N. 2002. A DNA-based method for identification of krill species and its application to analysing the diet of marine vertebrate predators. *Molecular Ecology*, 11, 2679-2690.
- JARMAN, S., REDD, K. & GALES, N. 2006. Group-specific primers for amplifying DNA sequences that identify Amphipoda, Cephalopoda, Echinodermata, Gastropoda, Isopoda, Ostracoda and Thoracica. *Molecular Ecology Notes*, 6, 268-271.
- JARMAN, S. & WILSON, S. 2004. DNA-based species identification of krill consumed by whale sharks. *Journal of Fish Biology*, 65, 586-591.
- JERNAKOFF, P., PHILLIPS, B. & FITZPATRICK, J. 1993. The diet of post-juvenile western rock lobster, *Panulirus cygnus* George, at Seven Mile Beach, Western Australia. *Australian Journal of Marine and Freshwater Research*, 44, 649-655.
- JOHNSON, C., BANKS, S., BARRETT, N., CAZZASUS, F., DUNSTAN, P., EDGAR, G., FRUSHER, S., GARDNER, C., HELIDONIOTIS, F., HILL, K., HOLBROOK, N., HOSIE, G., LAST, P., LING, S., MELBOURNE-THOMAS, J., MILLER, K., PECL, G., RICHARDSON, A., RIDGWAY, K., RINTOUL, S., RITZ, D., ROSS, D., SANDERSON, J., SHEPHERD, S., SLOTWINSKI, A., SWADLING, K. & TAW, N. 2011. Climate change cascades: shifts in oceanography, species' ranges and marine community dynamics in eastern Tasmania. *Journal of Experimental Marine Biology and Ecology* 400, 17-32.
- JOHNSON, C., LING, S., ROSS, J., SHEPHERD, S. & MILLER, K. 2005. Establishment of the long-spined sea urchin (*Centrostephanus rodgersii*) in Tasmania: first assessment of potential threats to fisheries.: FRDC Final Report 2001/044.
- JOHNSON, C., LING, S., SANDERSON, J., FLUKES, E., FRUSHER, S., GARDNER, C., HARTMANN, K., JARMAN, S., LITTLE, R., MARZLOFF, M., SOULIE, J., MELBOURNE-THOMAS, J. & REDD, K. 2012. Final report on FRDC 2007/045: Rebuilding Ecosystem Resilience: Assessment of management options to minimise formation of 'barrens' habitat by the long-spined sea urchin (*Centrostephanus rodgersii*) in Tasmania. *FRDC 2007/045*. Hobart: Institute of Marine and Antarctic Studies.
- JOHNSON, C., LING, S., SANDERSON, J. & REDD, K. in preparation. Final report on FRDC 2007/045: Rebuilding Ecosystem Resilience-Assessment of management options to minimize

- formation of “barrens” habitat by the long-spined sea urchin (*Centrostephanus rodgersii*) in Tasmania. Hobart: Institute of Marine and Antarctic Studies.
- JOHNSON, C., VALENTINE, J. & PEDERSON, H. A most unusual barrens: Complex interactions between lobsters, sea urchins and algae facilitates spread of an exotic kelp in eastern Tasmania. *In*: HEINZELLER, T. & NEBELSICK, J., eds. 11th International Echinoderm Conference, 2004 München. Balkema, Leiden, 213-220.
- JOLL, L. & PHILLIPS, B. 1984. Natural diet and growth of juvenile western rock lobsters *Panulirus cygnus* George. *Journal of Experimental Marine Biology and Ecology*, 75, 145-169.
- JOYCE, W., CAMPANA, S., NATANSON, L., KOHLER, N., PRATT, H. J. & JENSEN, C. 2002. Analysis of stomach contents of the porbeagle shark (*Lamna nasus Bonnatere*) in the Northwest Atlantic. *ICES journal of marine science*, 59, 1263-1269.
- JUEN, A. & TRAUGOTT, M. 2005. Detecting predation and scavenging by DNA gut-content analysis: a case study using a soil insect predator-prey system. *Oecologia*, 142, 344-352.
- JUEN, A. & TRAUGOTT, M. 2006. Amplification facilitators and multiplex PCR: tools to overcome PCR-inhibition in DNA-gut content analysis of soil-living invertebrates. *Soil Biology and Biochemistry*, 38, 1872-1879.
- KAMLER, J. & POPE, K. 2001. Nonlethal Methods of Examining Fish Stomach Contents. *Reviews in Fisheries Science*, 9, 1-11.
- KARTZINEL, T. R. & PRINGLE, R. M. 2015. Molecular detection of invertebrate prey in vertebrate diets: trophic ecology of Caribbean island lizards. *Molecular Ecology Resources*, 15, 903-914.
- KASPER, M., REESON, A., COOPER, S., PERRY, K. & AUSTIN, A. 2004. Assessment of prey overlap between a native (*Polistes humilis*) and an introduced (*Vespula germanica*) social wasp using morphology and phylogenetic analysis of 16s rDNA. *Molecular Ecology*, 13, 2037-2049.
- KEAR, A. 1992. The diet of Antarctic squid: Comparison of conventional and serological gut contents analyses. *Journal of Experimental Marine Biology and Ecology*, 156, 161-178.
- KELLY, K. & CHISTOSERDOV, A. 2001. Phylogenetic analysis of the succession of bacterial communities in the Great South Bay (Long Island). *FEMS Microbiology Ecology*, 35, 85-95.
- KELLY, S., SCOTT, D., MACDIARMID, A. & BABCOCK, R. 2000. Spiny lobster, *Jasus edwardsii*, recovery in New Zealand marine reserves. *Biological Conservation*, 92, 359-369.
- KING, R., READ, D., TRAUGOTT, M. & SYMONDSON, W. 2008a. Molecular analysis of predation: A review of best practice for DNA-based approaches. *Molecular Ecology*, 17, 947-963.
- KING, R., READ, D., TRAUGOTT, M. & SYMONDSON, W. 2008b. Molecular analysis of predation: A review of best practice for DNA-based approaches. *Molecular Ecology*, 17, 947-963.
- KING, R., VAUGHAN, I., BELL, J., BOHAN, D. & SYMONDSON, W. 2010. Prey choice by carabid beetles feeding on an earthworm community analysed using species- and lineage-specific PCR primers. *Molecular Ecology*, 19, 1721-1732
- LANGE, M., WESTERMANN, P. & AHRING, B. 2005. Archaea in protozoa and metazoa *Applied Microbiology and Biotechnology* 66, 465-474.
- LANGLOIS, T., ANDERSON, M. & BABCOCK, R. 2005. Reef-associated predators influence adjacent soft-sediment communities. *Ecology*, 86, 1508-1519.
- LANGLOIS, T., ANDERSON, M., BROCK, M. & MURMAN, G. 2006. Importance of rock lobster size-structure for trophic interactions: Choice of soft-sediment bivalve prey. *Marine Biology*, 149, 447-454.
- LEA, M.-A., CHEREL, Y., GUINET, C. & NICHOLS, P. 2002. Antarctic fur seals foraging in the Polar Frontal Zone: Inter-annual shifts in diet as shown from fecal and fatty acid analyses. *Marine Ecology Progress Series*, 245, 281-297.
- LESTER, S., HALPERN, B., GRORUD-COLVERT, K., LUBCHENCO, J., RUTTENBERG, B., GAINES, S. & AIRAMÉ, S. 2009. Biological Effects Within No-Take Marine Reserves: A global Synthesis. *Marine Ecology Progress Series*, 384, 33 - 46.

- LIGHT, R., ADLER, P. & ARNOLD, D. 1983. Evaluation of gastric lavage for stomach analyses. *North American Journal of Fisheries Management*, 3, 81-85.
- LING, S. 2008. Range expansion of a habitat-modifying species leads to loss of taxonomic diversity: a new and impoverished reef state. *Oecologia*, 156, 883-894.
- LING, S. 2009. *RE: Observations of rock lobster foraging behaviour after translocation*. Type to REDD, K.
- LING, S., IBBOTT, S. & SANDERSON, J. 2010. Recovery of canopy-forming macroalgae following removal of the enigmatic grazing sea urchin *Heliocidaris erythrogramma*. *Journal of Experimental Marine Biology & Ecology*, 395, 135-146.
- LING, S. & JOHNSON, C. 2009. Population dynamics of an ecologically important range extender: kelp beds vs. sea urchin barrens. *Marine Ecology Progress Series*, 374, 113-125.
- LING, S. & JOHNSON, C. 2012. Marine reserves reduce risk of climate-driven phase shift by restoring size and habitat specific trophic interactions. *Ecological Applications*, 22.
- LING, S., JOHNSON, C., FRUSHER, S. & RIDGWAY, K. 2009a. Overfishing reduces resilience of kelp beds to climate-driven catastrophic phase shift *Proceedings of the National Academy of Sciences of the United States of America*, 106, 22341-22345
- LING, S., JOHNSON, C., RIDGWAY, K., HOBDAI, A. & HADDON, M. 2009b. Climate driven range extension of a sea urchin: inferring future trends by analysis of recent population dynamics. *Global Change Biology* 15, 719-731.
- LIU, J., LI, Q., KONG, L., YU, H. & ZHENG, X. 2011. Identifying the true oysters (Bivalvia: Ostreidae) with mitochondrial phylogeny and distance-based DNA barcoding. *Molecular Ecology Resources*, 11, 820-830.
- LORD, J. P. & DALVANO, B. E. 2015. Differential response of the American lobster *Homarus americanus* to the invasive Asian shore crab *Hemigrapsus sanguineus* and green crab *Carcinus maenas*. *Journal of Shellfish Research*, 34, 1091-1096.
- LORDAN, C., COLLINS, M. & PERALES-RAYA, C. 1998. Observations on morphology, age and diet of three *Architeuthis* caught off the west coast of Ireland in 1995. *Journal of the Marine Biological Association of the United Kingdom*, 78, 903-917.
- MACDIARMID, A., HICKEY, B. & MALLER, R. 1991. Daily movement patterns of the spiny lobster *Jasus edwardsii* (Hutton) on a shallow reef in northern New Zealand. *Journal of Experimental Marine Biology and Ecology*, 147, 185-205.
- MARTIN, D., ROSS, R., QUETIN, L. & MURRAY, A. 2006. Molecular approach (PCR-DGGE) to diet analysis in young Antarctic krill *Euphausia superba*. *Marine Ecology Progress Series*, 319, 155-165.
- MATTHEE, C., COCKCROFT, A., GOPAL, K. & VON DER HEYDEN, S. 2007. Mitochondrial DNA variation of the west-coast rock lobster, *Jasus lalandii*: marked genetic diversity differences among sampling sites. *Marine and Freshwater Research* 58, 1130-1135
- MAYFIELD, S., ATKINSON, L., BRANCH, G. & COCKCROFT, A. 2000a. Diet of the west coast rock lobster *Jasus lalandii*: Influence of lobster size, sex, capture depth, latitude and moult stage. *South African Journal of Marine Science*, 22, 57-69.
- MAYFIELD, S. & BRANCH, G. 2000. Interrelations among rock lobsters, sea urchins, and juvenile abalone: implications for community management. *Canadian Journal of Fisheries and Aquatic Sciences*, 57, 2175-2185.
- MAYFIELD, S., BRANCH, G. & COCKCROFT, A. 2000b. Relationships among diet, growth rate, and food availability for the South African rock lobster, *Jasus lalandii* (Decapoda, Palinuridea). *Crustaceana*, 73, 815-834.
- MAYFIELD, S., DE BEER, E. & BRANCH, G. 2001. Prey preference and the consumption of sea urchins and juvenile abalone by captive rock lobsters (*Jasus lalandii*). *Marine & Freshwater Research*, 52, 773-780.

- MAYFIELD, S., LOPATA, A. & BRANCH, G. 2000c. Limitation and failure of immunological technique (ELISA) in resolving the diet of the South African rock lobster *Jasus lalandii*. *Marine Biology*, 137, 595-604.
- MCCLANAHAN, T. & SHAFIR, S. 1990. Causes and consequences of sea urchin abundance and diversity in Kenyan coral reef lagoons. *Oecologia*, 83, 362-370.
- MCKEMEY, A., SYMONDSON, W. & GLEN, D. 2003. Predation and prey size choice by the carabid beetle *Pterostichus melanarius* (Coleoptera: Carabidae): the dangers of extrapolating from laboratory to field. *Bulletin of Entomological Research*, 93, 227-234.
- MCMAHAN, M. D., BRADY, D. C., COWAN, D. F., GRABOWSKI, J. H. & SHERWOOD, G. D. 2013. Using acoustic telemetry to observe the effects of a groundfish predator (Atlantic cod, *Gadus morhua*) on movement of the American lobster (*Homarus americanus*). *Canadian Journal of Fisheries and Aquatic Sciences*, 70, 1625-1634.
- MCPHERSON, M. J. 1991. *PCR, a practical approach*, Oxford, IRL Press at Oxford University Press.
- MICHELI, F., AMARASEKARE, P., BASCOMPTE, J. & GERBER, L. 2004. Including Species Interactions in the Design and Evaluation of Marine Reserves: Some Insights from a Predator-prey Model. *Bulletin of Marine Science*, 74, 653-669.
- MILLS, D., GARDNER, C. & JOHNSON, C. 2006. Experimental reseedling of juvenile spiny lobsters (*Jasus edwardsii*): Comparing survival and movement of wild and naïve lobsters at multiple sites. *Aquaculture*, 254, 256 - 268.
- MOBIOLABORATORIES 2003. UltraClean Soil DNA Isolation Kit: Instruction Manual. *Technical Support*. Mo Bio Laboratories, Inc.
- MOLONEY, C., ST JOHN, M., DENMAN, K., KARL, D., KÖSTER, F., SUNDBY, S. & WILSON, R. 2011. Weaving marine food webs from end to end under global change *Journal of Marine Systems*, 84, 106-116
- MONTRESOR, M., NUZZO, L. & MAZZOCCHI, M. 2003. Viability of dinoflagellate cysts after the passage through the copepod gut *Journal of Experimental Marine Biology and Ecology*, 287, 209-221
- MUMMA, M. A., ADAMS, J. R., ZIEMINSKI, C., FULLER, T. K., MAHONEY, S. P. & WAITS, L. P. 2016. A comparison of morphological and molecular diet analyses of predator scats. *Journal of Mammalogy*, 97, 112-120.
- MURAJI, M., KAWASAKI, K., SHIMIZU, T. & NODA, T. 2004. Discrimination among Japanese species of the Orius Flower Bugs (Heteroptera: Anthracoridae) Based on PCR-RFLP of the Nuclear and Mitochondrial DNAs. *Japan Agriculture Research Quarterly*, 38, 91-95.
- NCBI. 2006. *GenBank* [Online]. National Center for Biotechnology Information. Available: <http://www.ncbi.nih.gov/Genbank/> [Accessed 1 March 2006].
- NEJSTGAARD, J., FRISCHER, M., RAULE, C., GRUEBEL, R., KOHLBERG, K. & VERITY, P. 2003. Molecular detection of algal prey in copepod guts and fecal pellets. *Limnology and Oceanography: Methods*, 1, 29-38.
- NEJSTGAARD, J., FRISCHER, M., SIMONELLI, P., TROEDSSON, C., BRAKEL, M., ADIYAMAN, F., SAZHIN, A. & ARTIGAS, F. 2008. Quantitative PCR to estimate copepod feeding. *Marine Biology*, 153, 565-577.
- NIELSEN, R. & MATZ, M. 2005. Statistical approaches to DNA barcoding. *Systematic Biology*, 55, 162-169.
- NORMAN-LOPEZ, A., PASCOE, S., THÉBAUD, O., VAN PUTTEN, I., INNES, J., JENNINGS, S., HOBDA, A., GREEN, B. & PLAGANYI, E. 2014. Price integration in the Australian rock lobster industry: implications for management and climate change adaptation. *Australian Journal of Agricultural and Resource Economics*, 58, 43-59.
- NYSSSEN, F., BREY, T., DAUBY, P. & GRAEVE, M. 2005. Trophic position of Antarctic amphipods—enhanced analysis by a 2-dimensional biomarker assay. *Marine ecology progress series*, 300, 135-145.

- O'MEARA, D. B., SHEEHY, E., TURNER, P. D., O'MAHONY, D., HARRINGTON, A. P., DENMAN, H., LAWTON, C., MACPHERSON, J. & O'REILLY, C. 2014. Non-invasive multi-species monitoring: real-time PCR detection of small mammal and squirrel prey DNA in pine marten (*Martes martes*) scats. *Acta Theriologica*, 59, 111-117.
- PANDE, A., MACDIARMID, A., SMITH, P., DAVIDSON, R., COLE, R., FREEMAN, D., KELLY, S. & GARDNER, J. 2008. Marine reserves increase the abundance and size of blue cod and rock lobster. *Marine Ecology Progress Series*, 147-158.
- PARSONS, K., PIERTNEY, S., MIDDLEMAS, S., HAMMOND, P. & ARMSTRONG, J. 2005. DNA-based identification of salmonid prey species in seal faeces. *Journal of Zoology*, 266, 275-281.
- PASSMORE, A., JARMAN, S., SWADLING, K., KAWAGUCHI, S., MCMINN, A. & NICOL, S. 2006. DNA as a dietary biomarker in Antarctic krill, *Euphausia superba*. *Marine Biotechnology*, 8, 686-696.
- PEARCE-HIGGINS, J. 2010. Using diet to assess the sensitivity of northern and upland birds to climate change. *Climate Research*, 45, 119-130.
- PEDERSON, H. & JOHNSON, C. 2006. Predation of the sea urchin *Heliocidaris erythrogramma* by rock lobsters (*Jasus edwardsii*) in no-take marine reserves. *Journal of Experimental Marine Biology and Ecology*, 336, 120-134.
- PEGARD, A., MIQUEL, C., VALENTINI, A., COISSAC, E., BOUVIER, F., FRANÇOIS, D., TABERLET, P., ENGEL, E. & POMPANON, F. 2009. Universal DNA-Based Methods for Assessing the Diet of Grazing Livestock and Wildlife from Feces. *Journal of agricultural and food chemistry*, 57, 5700 - 5706.
- PEREZ-BARROS, P., ROMERO, M. C., CALCAGNO, J. A. & LOVRICH, G. A. 2010. Similar feeding habits of two morphs of *Munida gregaria* (Decapoda) evidence the lack of trophic polymorphism. *Revista De Biología Marina Y Oceanografía*, 45, 461-470.
- PERKEL, J. 2006. Five questions on qPCR. *Scientist*, 20, 66-67.
- PETERS, K. J., OPHELKELLER, K., HERDINA, BOTT, N. J. & GOLDSWORTHY, S. D. 2015. PCR-based techniques to determine diet of the Australian sea lion (*Neophoca cinerea*): a comparison with morphological analysis. *Marine Ecology-an Evolutionary Perspective*, 36, 1428-1439.
- PHILLIPS, K., JACKSON, G. & NICHOLS, P. 2001. Predation on myctophids by the squid *Moroteuthis ingens* around Macquarie and Heard Islands: Stomach contents and fatty acid analysis. *Marine Ecology Progress Series*, 215, 179-189.
- PHILLIPS, K., NICHOLS, P. & JACKSON, G. 2003. Size-related dietary changes observed in the squid *Moroteuthis ingens* at the Falkland Islands: stomach contents and fatty-acid analyses. *Polar Biology*, 26, 474 - 485.
- PIERCE, G. & BOYLE, P. 1991. A review of methods for diet analysis in piscivorous marine mammals. *Oceanography and marine biology: an annual review.*, 29, 409-486.
- PINEAU, P., HENRY, M., SUSPENE, R., MARCHIO, A., DETTAI, A., DEBRUYNE, R., PETIT, T., LECU, A., MOISSON, P., DEJEAN, A., WAIN-HOBSON, S. & VARTANIAN, J. 2005. A Universal Primer Set for PCR Amplification of Nuclear Histone H4 Genes from All Animal Species. *Molecular Biology and Evolution*, 22, 582-588.
- PONS, J. 2006. DNA-based identification of preys from non-destructive, total DNA extractions of predators using arthropod universal primers. *Molecular Ecology Notes*, 6, 623-626.
- PURCELL, M., MACKAY, G., LAHOOD, E., HUBER, H. & PARK, L. 2004. Molecular methods for the genetic identification of salmonid prey from Pacific harbor seal (*Phoca vitulina richardsi*) scat. *Fishery Bulletin*, 102, 213-220.
- QIAGEN. 2004. *DNeasy Tissue Handbook* [Online]. QIAGEN, Pty, Ltd. Available: www.qiagen.com [Accessed March 2004].
- QIAGEN. 2008. *QIAamp DNA Micro Handbook* [Online]. Technical Support.
- QUINCE, C., LANZEN, A., CURTIS, T., DAVENPORT, R., HALL, N., HEAD, I., READ, L. & SLOAN, W. 2009. Accurate determination of microbial diversity from 454 pyrosequencing data. *Nature Methods*, 6, 639-641.

- QUINCE, C., LANZEN, A., DAVENPORT, R. & TURNBAUGH, P. 2011. Removing noise from pyrosequenced amplicons. *BMC Bioinformatics*, 12, 38.
- RACLOT, T., GROSCOLAS, R. & CHEREL, Y. 1998. Fatty acid evidence for the importance of myctophid fishes in the diet of king penguins, *Aptenodytes patagonicus*. *Marine Biology*, 132, 523-533.
- RAPPE, M., KEMP, P. & GIOVANNONI, S. 1997. Phylogenetic diversity of marine coastal picoplankton 16S rRNA genes cloned from the continental shelf off Cape Hatteras, North Carolina. *Limnology and Oceanography*, 42, 811-826.
- RATNASINGHAM, S. & HEBERT, P. 2007. *BOLD Identification System* [Online]. University of Guelph.
- RAYE, G., MIQUEL, C. & COISSAC, E. 2011. New insights on diet variability revealed by DNA barcoding and high-throughput pyrosequencing: chamois diet in autumn as a case study *Ecological research*, 26, 265 - 276.
- READ, D., SHEPPARD, S., BRUFORD, M., GLEN, D. & SYMONDSON, W. 2006. Molecular detection of predation by soil micro-arthropods on nematodes. *Molecular Ecology*, 15, 1963-1972.
- REDD, K., JARMAN, S., FRUSHER, S. & JOHNSON, C. 2008. A molecular approach to identify prey of the southern rock lobster. *Bulletin of Entomological Research*, 98, 233-238.
- REÑONES, O., POLUNIN, N. & GONI, R. 2002. Size related dietary shifts of *Epinephelus marginatus* in a western Mediterranean littoral ecosystem: an isotope and stomach content analysis. *Journal of Fish Biology*, 61, 122-137.
- ROESCH, L., FULTHORPE, R., RIVA, A., CASELLA, G., HADWIN, A., KENT, A., DAROUB, S., CAMARGO, F., FARMERIE, W. & TRIPLETT, E. 2007. Pyrosequencing enumerates and contrasts soil microbial diversity *The ISME Journal*, 1, 283 - 290.
- ROSAS-LUIS, R., SANCHEZ, P., PORTELA, J. M. & DEL RIO, J. L. 2014. Feeding habits and trophic interactions of *Doryteuthis gahi*, *Illex argentinus* and *Onykia ingens* in the marine ecosystem off the Patagonian Shelf. *Fisheries Research*, 152, 37-44.
- ROSEL, P. & KOCHER, T. 2002. DNA-based identification of larval cod in stomach contents of predatory fishes. *Journal of Experimental Marine Biology and Ecology*, 267, 75-88.
- ROSLIN, T. & MAJANEVA, S. 2016. The use of DNA barcodes in food web construction-terrestrial and aquatic ecologists unite! *Genome*, 59, 603-628.
- ROUALDES, E. A., BONNER, S. J., WHITNEY, T. D. & HARWOOD, J. D. 2016. Formal modelling of predator preferences using molecular gut-content analysis. *Environmental and Ecological Statistics*, 23, 317-336.
- ROUGEMONT, M., VAN SAANEN, M., SAHLI, R., HINRIKSON, H., BILLE, J. & JATON, K. 2004. Detection of Four Plasmodium Species in Blood from Humans by 18S rRNA Gene Subunit-Based and Species-Specific Real-Time PCR Assays. *Journal of Clinical Microbiology*, 42, 5636-5643.
- ROURA, Á., GONZÁLEZ, A., PASCUAL, S. & GUERRA, A. 2010. A molecular approach to identifying the prey of cephalopod paralarvae. *ICES Journal of Marine Science*, 67, 1408-1412.
- ROURA, Á., GUERRA, Á., REDD, K. & GONZÁLEZ, Á. 2012. Molecular prey identification in wild *Octopus vulgaris* paralarvae *Marine Biology*, 159, 1335-1345.
- RYCHLIK, W. 2007. OLIGO 7 primer analysis software. *Methods in Molecular Biology*, 402, 35-59.
- SATOKARI, R., VAUGHAN, E., SMIDT, H., SAARELA, M., MATTO, J. & DE VOS, W. 2003. Molecular approaches for the detection and identification of bifidobacteria and lactobacilli in the human gastrointestinal tract. *Systematic and Applied Microbiology*, 26, 572-584.
- SEGURA-GARCÍA, I., BRIONES-FOURZÁN, P., DE LESTANG, S. & LOZANO-ÁLVAREZ, E. 2016. Dietary partitioning between sympatric species of spiny lobster in a coral reef system. *Bulletin of Marine Science*, 92, 355-369.
- SHEARS, N. & BABCOCK, R. 2002. Marine reserves demonstrate top-down control of community structure on temperate reefs. *Oecologia*, 132, 131-142.
- SHEARS, N. & BABCOCK, R. 2003. Continuing trophic cascade effects after 25 years of no-take marine reserve protection. *Marine ecology progress series*, 246, 1-16.

- SHEPPARD, S., BELL, J., SUNDERLAND, K., FENLON, J., SKERVIN, D. & SYMONDSON, W. 2005. Detection of secondary predation by PCR analyses of the gut contents of invertebrate generalist predators. *Molecular Ecology*, 14, 4461-4468.
- SHEPPARD, S. & HARWOOD, J. 2005. Advances in molecular ecology: tracking trophic links through predator-prey food-webs. *Functional Ecology*, 19, 751-762.
- SHEPPARD, S., HENNEMAN, J., MEMMOTT, J. & SYMONDSON, W. 2004. Infiltration by alien predators into invertebrate food webs in Hawaii: a molecular approach. *Molecular Ecology*, 13, 2077-2089.
- SILVAR, C., PEROVIC, D., CASAS, A., IGARTUA, E. & ORDON, F. 2011. Development of a cost effective pyrosequencing approach for SNP genotyping in barley. *Plant Breeding*, 130, 394 - 397.
- SIMON, C., FRATI, F., BECKENBACH, A., CRESPI, B., LIU, H. & FLOOK, P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America*, 87, 651-701.
- SINT, D., RASO, L., KAUFMANN, R. & TRAUGOTT, M. 2011. Optimizing methods for PCR-based analysis of predation. *Molecular Ecology Resources*, 11, 795-801.
- SMITH, P., MCVEAGH, S., ALLAIN, V. & SANCHEZ, C. 2005. DNA identification of gut contents of large pelagic fishes. *Journal of Fish Biology*, 67, 1178-1183.
- STEYNA, E. & SCHLEYERA, M. 2011. Movement patterns of the East Coast rock lobster *Panulirus homarus rubellus* on the coast of KwaZulu-Natal, South Africa. *New Zealand Journal of Marine and Freshwater Research* 45, 85-101.
- STRAUSBERG, R., FEINGOLD, E., KLAUSNER, R. & COLLINS, F. 1999. The Mammalian Gene Collection. *Science*, 286, 455-457.
- SUZUKI, N., MURAKAMI, K., TAKEYAMA, H. & CHOW, S. 2006. Molecular attempt to identify prey organisms of lobster phyllosoma larvae. *Fisheries Science*, 72, 342-349.
- SYMONDSON, W. 2002. Molecular identification of prey in predator diets. *Molecular Ecology*, 11, 627-641.
- SYMONDSON, W. & LIDDELL, J. 1995. Decay rates for slug antigens within the carabid predator *Pterostichus melanarius* monitored with a monoclonal antibody. *Entomologia Experimentalis et Applicata*, 75, 245-250.
- TARR, R., WILLIAMS, P. & MACKENZIE, A. 1996. Abalone, sea urchins and rock lobster: A possible ecological shift that may affect traditional fisheries. *South African Journal of Marine Science*, 17, 319-323.
- TEGNER, M. & LEVIN, L. 1983a. Spiny lobsters and sea urchins: Analysis of a predator-prey interaction *Journal of Experimental Marine Biology and Ecology*, 73, 125 - 150.
- TEGNER, M. & LEVIN, L. 1983b. Spiny lobsters and sea urchins: analysis of a predator-prey interaction. *Journal of Experimental Marine Biology and Ecology*, 73, 125-150.
- THORNTON, B. & CHHANDAK, B. 2011. Real-Time PCR (qPCR) Primer Design Using Free Online Software. *Biochemistry and Molecular Biology Education*, 39, 145 - 154.
- TIEDE, J., WEMHEUER, B., TRAUGOTT, M., DANIEL, R., TSCHARNTKE, T., EBELING, A. & SCHERBER, C. 2016. Trophic and Non-Trophic Interactions in a Biodiversity Experiment Assessed by Next-Generation Sequencing. *Plos One*, 11.
- TRELOGGEN, R. 2009. *RE: Observations on rock lobster foraging behaviour: a fisherman's perspective*. Type to LING, S.
- TROEDSSON, C., FRISCHER, M., NEJSTGAARD, J. & THOMPSON, E. 2007. Molecular quantification of differential ingestion and particle trapping rates by the appendicularian *Oikopleura dioica* as a function of prey size and shape. *Limnology and Oceanography*, 52, 416-427.
- TWEEDIE, S., ASHBURNER, M., FALLS, K., LEYLAND, P., MCQUILTON, P., MARYGOLD, S., MILLBURN, G., OSUMI-SUTHERLAND, D., SCHROEDER, A., SEAL, R., ZHANG, H. & CONSORTIUM, T. F.

2009. FlyBase: enhancing Drosophila Gene Ontology annotations. *Nucleic Acids Research*, 37, 555-559.
- VAN ZYL, R., MAYFIELD, S., PULFRICH, A. & GRIFFITHS, C. 1998. Predation by West Coast rock lobsters (*Jasus lalandii*) on two species of wrinkle (*Oxystele sinensis* and *Turbo cidaris*). *South African Journal of Zoology*, 33, 203-209.
- VENCES, M., THOMAS, M., VAN DER MEIJDEN, A., CHIARI, Y. & VIEITES 2005. Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. *Frontiers in Zoology*, 2.
- VESTHEIM, H., EDVARDSEN, B. & KAARTVEDT, S. 2005. Assessing feeding of a carnivorous copepod using species-specific PCR. *Marine Biology*, 147, 381-385.
- VESTHEIM, H. & JARMAN, S. 2008. Blocking primers to enhance PCR amplification of rare sequences in mixed samples - A case study on prey DNA in Antarctic krill stomachs. *Frontiers in Zoology*, 5, art 12.
- VILLANUEVA, R. & NORMAN, M. 2008. Biology of the planktonic stages of benthic octopuses. *Oceanography and marine biology: an annual review.*, 46, 105.
- WABNITZ, C., BALAZS, G., BEAVERS, S., BJORNDALE, K., BOLTON, A., CHRISTENSEN, V., HARGROVE, S. & PAULY, D. 2010. Ecosystem structure and processes at Kaloko Honokōhau, focusing on the role of herbivores, including the green sea turtle *Chelonia mydas*, in reef resilience. *Marine Ecology Progress Series* 420, 27-44.
- WADDINGTON, K., BELLCHAMBERS, L., VANDERKLIFT, M. & WALKER, D. 2008. Western rock lobsters (*Panulirus cygnus*) in Western Australian deep coastal ecosystems (35–60m) are more carnivorous than those in shallow coastal ecosystems. *Estuarine, Coastal and Shelf Science*, 79, 114-120.
- WALDNER, T. & TRAUGOTT, M. 2012. DNA-based analysis of regurgitates: a noninvasive approach to examine the diet of invertebrate consumers. *Molecular Ecology Resources*, 12, 669-675.
- WARD, R., ZEMLAK, T., INNES, B., LAST, P. & HEBERT, P. 2005. DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society of London*, 360, 1847-1857.
- WARD, T., HEINEMANN, D. & EVANS, N. 2001. The role of marine reserves as fisheries management tools: a review of concepts, evidence and international experience. In: SCIENCES, B. O. R. (ed.). A.C.T. (Australia): Department of Agriculture, Fisheries and Forestry.
- WATSON, J. & ESTES, J. 2011. Stability, resilience, and phase shifts in rocky subtidal communities along the west coast of Vancouver Island, Canada. *Ecological Monographs*, 81, 215–239.
- WEBER, D. & LUNDGREN, J. 2009a. Detection of predation using qPCR: Effect of prey quantity, elapsed time, chaser diet, and sample preservation on detectable quantity of prey DNA. *Journal of Insect Science*, 9, 12.
- WEBER, D. & LUNDGREN, J. 2009b. Detection of predation using qPCR: Effect of prey quantity, elapsed time, chaser diet, and sample preservation on detectable quantity of prey DNA. *Journal of Insect Science*, 9, 41.
- WELLS, M. 1978. *Octopus : physiology and behaviour of an advanced invertebrate*, London, Chapman and Hall.
- WERNBERG, T., RUSSELL, B., MOORE, P., LING, S., SMALE, D., CAMPBELL, A., COLEMAN, M., STEINBERG, P., KENDRICK, G. & CONNELL, S. 2011. Impacts of climate change in a global hotspot for temperate marine biodiversity and ocean warming. *Journal of Experimental Marine Biology and Ecology*, 400, 7-16.
- WHITE, G. & BURNHAM, K. 1999. Program MARK: survival estimation from populations of marked animals. *Bird study*, 46, S120-S139.
- WILLIAMS, M. 1981. Methods for Analysis of Natural Diet in Portunid Crabs (Crustacea:Decapoda:Portunidae). *Journal of Experimental Marine Biology and Ecology*, 52, 103-113.
- WILSON, A. K. 1999. *Encyclopedia of Molecular Biology*, New York, Wiley.

- XIONG, M. Y., SHAO, X. N., LONG, Y., BU, H. L., ZHANG, D., WANG, D. J., LI, S., WANG, R. J. & YAO, M. 2016. Molecular analysis of vertebrates and plants in scats of leopard cats (*Prionailurus bengalensis*) in southwest China. *Journal of Mammalogy*, 97, 1054-1064.
- ZAIDI, R., JAAL, Z., HAWKES, N., HEMINGWAY, J. & SYMONDSON, W. 1999. Can multiple-copy sequences of prey DNA be detected amongst the gut contents of invertebrate predators? *Molecular Ecology*, 8, 2081-2087.
- ZARZOSO-LACOSTE, D., BONNAUD, E., CORSE, E., GILLES, A., MEGLECZ, E., COSTEDOAT, C., GOUNI, A. & VIDAL, E. 2016. Improving morphological diet studies with molecular ecology: An application for invasive mammal predation on island birds. *Biological Conservation*, 193, 134-142.
- ZARZOSO-LACOSTE, D., CORSE, E. & VIDAL, E. 2013. Improving PCR detection of prey in molecular diet studies: importance of group-specific primer set selection and extraction protocol performances. *Molecular Ecology Resources*, 13, 117-127.
- ZEALE, M., BUTLIN, R., BARKER, G., LEES, D. & JONES, G. 2011. Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. *Molecular Ecology Resources*, 11, 236-244.
- ZHU, Y.-C., BURD, J., ELLIOTT, N. & GREENSTONE, M. 2000. Specific Ribosomal DNA Marker for Early Polymerase Chain Reaction Detection of *Aphelinus hordei* (Hymenoptera: Aphelinidae) and *Aphidius colemani* (Hymenoptera: Aphididae) from *Diuraphis noxia* (Homoptera: Aphididae). *Ecology and Population Biology*, 93, 486-491.
- ZHU, Y.-C. & GREENSTONE, M. 1999. Polymerase Chain Reaction Techniques for Distinguishing Three Species and Two Strains of *Aphelinus* (Hymenoptera: Aphelinidae) from *Diuraphis noxia* and *Schizaphis graminum* (Homoptera: Aphididae). *Annals of the Entomological Society of America*, 92, 71-79.
- ZIEGLER, P., FRUSHER, S. & JOHNSON, C. 2003. Space-time variation in catchability of southern rock lobster *Jasus edwardsii* in Tasmania explained by environmental, physiological and density-dependent processes. *Fisheries Research*, 61, 107-123.